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Title:
Gene expression of Neisseria meningitidis in the upper respiratory tract

Gene expression of *Neisseria meningitidis* in the upper respiratory tract

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Doctor of Philosophy in the Faculty of Life Sciences

School of Cellular and Molecular Medicine

June 2019

Word count 30,358

Abstract

Transcriptomic analysis of pharyngeal swab samples from carriers of *Neisseria meningitidis* (Nm) may help to predict whether protein antigen meningococcal vaccines might impact on herd protection. Although RNA extraction and detection of Nm gene transcripts from *in vivo* mucosal samples is challenging, a probe-based gene expression profiling platform, the NanoString nCounter system, can detect very low numbers of messenger RNA (mRNA) transcripts.

RNA extraction methods were compared. The RNeasy mini kit method was easier to perform, gave more reproducible results, and delivered a higher quality and quantity of RNA than the alternative acid:phenol:chloroform method tested.

A panel of 47 Nm genes were selected for NanoString probe design that coded for proteins in current or potential vaccine candidates or that were important in colonisation of the pharyngeal mucosa.

The NanoString system was first assessed in Nm culture samples. The method showed high sensitivity of gene detection and high reproducibility. Exposure to heat, cold and iron depletion resulted in a range of gene expression across the panel. This study showed that mRNA can be successfully detected and quantified from Nm cultures without the need to amplify the genes and from very small quantities of total RNA.

In subsequent experiments, Nm genes were detected and counted from *in vivo* pharyngeal carriage samples for the first time. Isolates from the samples were whole genome sequenced to assess the gene expression results obtained by NanoString. Some limitations of the technique were demonstrated including cross reactivity between different species in samples containing multiple organisms and reduced specificity at low levels of gene expression. The methodologies established in this thesis should be important for future transcriptomic studies on Nm and other bacteria carried in the human pharynx, and for the assessment of actual and potential vaccine antigens.

Dedication

I dedicate this PhD to my son Kidus

Acknowledgments

Without these people, this thesis could not have been completed. Firstly, and foremost I would like to thank my husband James and my son Kidus for their love, support and continuous encouragement. Thank you, James, for your guidance in writing this thesis and giving me the time I needed to do my work. Gratitude is also extended to my Ethiopian family, my mother, sisters, brothers and in laws.

I am very grateful to the Finn research group (especially Begonia, Liz, Kaltun, Paulina and Kirsty) and Darryl Hill's team (Darryl, Clio and Matt) for all their support in the laboratory. I would like to thank Rosy Reynolds for her help with statistical analysis. I am grateful to Christopher Bayliss, Caroline Caryou and Luke Green for sharing their knowledge and assisting in processing my samples at the University of Leicester laboratory. I would like to thank Jim White from NanoString for his help and letting me contact him at any time of the day.

I would like to thank Jenny Oliver who encouraged me to apply for this PhD. I appreciate the help of Jay Lucidarme and Ray Borrow of Public Health England in sequencing my samples. I would like to thank Kanny Diallo, Keith Jolley and Odile Harrison at the University of Oxford for their advice and support.

I would like to thank all my friends, particularly Musa-Hassan-King, Diana, Cilia, Mulu and Cynthia for their constant moral support and being with me all the way. I would like to thank my internal progress panels Jim Spencer and Tom Gaunt for their constructive comments and encouragement. I am grateful to the staff of the Health Protection Research Unit, especially Matt Hickman and Gemma Lasseter, for their constant support. I would like to acknowledge Cathleen Schmitke for her welcoming smile and quick response whenever I needed administrative help.

Finally, I would like to thank the two special people who particularly gave me the confidence, support, encouragement to do this PhD. Thank you for being understanding and appreciative of my work. You are both no longer my supervisors but good friends. Thank you, Adam and Hannah.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE: .....

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Abbreviations

µl	Microlitre
ATCC	American Type Culture Collection
CBA	Colombian blood agar
cDNA	Complementary DNA
CO_2	Carbon dioxide
CSV	Comma separated value
DNA	Deoxyribonucleic acid
ERCC	External RNA Control Consortium
FOV	Field of view
g	Gram
g	Units of centrifugal force
H_2O	Water
BHI	Brain heart infusion
IGR	Intergenic region
IMD	Invasive meningococcal disease
M	Molar
MenB	Serogroup B Nm
mg	Milligram
ml	Millilitre

mRNA	Messenger RNA
NIHR	National Institute for Health Research
Nm	*Neisseria meningitidis*
PBS	Phosphate buffered saline
PBS-B	Phosphate buffered saline with calcium and magnesium
HPRU	Health Protection Research Unit
PCR	Polymerase chain reaction
PHE	Public Health England
QC	Quality control
qPCR	Quantitative PCR
RCC	Reporter code count
RLF	Reporter library file
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNA-Seq	RNA sequencing
rpm	Revolutions per minute
RT-qPCR	Reverse transcriptase qPCR
STGG	Skim milk, tryptone, glucose, and glycerine
UoB	University of Bristol
WGS	Whole genome sequencing

Chapter 1. Introduction

1.1 Context

This PhD was set up within the National Institute for Health Research (NIHR) Heath Protection Research Unit (HPRU) at the University of Bristol (UoB) as part of a programme of research into carriage of *Neisseria meningitidis* (Nm), the meningococcus. This bacterium is commonly carried harmlessly in the human pharynx but can cause invasive meningococcal disease (IMD), mainly presenting as meningitis and septicaemia. Serogroup B (MenB) was the predominant cause of disease in the UK, and a new MenB meningococcal vaccine containing four protein antigens (Bexsero) was soon to be added to the UK universal primary schedule for infants. It was not known whether this vaccine would impact on acquisition or clearance of carriage. This would probably depend upon levels of expression of the vaccine antigens by the bacteria during colonisation. Any effect on reducing carriage is important as it may lead to indirect protection of unvaccinated groups (herd protection).

The HPRU was currently conducting a longitudinal study to detect and quantify meningococcal pharyngeal carriage in 16 to 18-year-old students. Swabs had been stored in ribonucleic acid (RNA) preservation medium. Direct measurement of levels of protein antigen expression by bacteria sampled from the pharynx is difficult even when relatively large numbers of bacteria are present. The intention of this PhD was to use novel transcriptomic techniques to study gene expression by meningococci in

pharyngeal carriage samples, and to gain insight into the potential impact of actual or potential vaccine protein antigens on the carriage of meningococci.

1.2 Chapter overview

Chapter 2 details the background to the thesis, including the meningococcus, pharyngeal carriage, meningococcal vaccines, and transcriptomics.

The different materials and methods used to perform the assays are described in Chapter 3, as well as the quality control (QC) and data analysis methods used to assess gene expression.

In Chapter 4, the different validation tests used to extract total RNA and assess the use of the NanoString nCounter gene profiling system for Nm culture samples are described. Two RNA extraction methods, one more modern technique (RNeasy mini kit) and another more traditional technique (acid:phenol:chloroform) are compared. Extraction of RNA using RNeasy is compared with extraction directly from lysate. RNA extraction using PrimeStore RNA collection, storage and extraction kits is compared with extraction from samples collected with RNAlater using the RNeasy minikit. The sensitivity, specificity and reproducibility of the NanoString nCounter technology is evaluated on Nm cultures, and gene profiling is assessed under hot, cold, and iron deficient conditions.

The use of NanoString technology for transcript detection of gene expression of Nm from *in vivo* carriage samples is evaluated in Chapter 5, the first time that this has been attempted. The second part of Chapter 5 shows the validation the NanoString result for one gene (*fhbP*) using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).

In Chapter 6, the presence or absence of 47 Nm genes in the genome of Nm carriage isolates is assessed by whole genome sequencing (WGS). The level of expression of *fhbP* is assessed using intergenic region (IGR) identification and compared with expected levels from previous studies. Genetic diversity is measured in paired longitudinal carriage samples.

In Chapter 7, the overall results of the thesis are discussed as well as ideas for future research.

1.3 Publications and presentations

This work has been subject to peer review and shared with the academic community on several occasions. Work from Chapters Three, Four and Five has been presented.

One paper (Appendix A) based on the work described in Chapter Three was published in 2018: Tekletsion YK, Christensen H, Finn A. Gene detection and expression profiling of *Neisseria meningitidis* using NanoString nCounter platform. J Microbiol Methods. 2018; 146:100-103. (doi):10.1016/j.mimet.2018.02.003. Epub Feb 7 (1)

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Two more papers are in preparation, the first is based on the work in Chapter Five and the second based on the work in Chapter Six: "Tekletsion YK, Christensen H, Cayrou C, Bayliss C, Reynolds R, Oliver J, Morales-Aza B, Rodrigues F, Finn A. Gene expression profiling of *Neisseria meningitidis* from pharyngeal carriage samples", and "Tekletsion YK, Christensen H, Lucidarme J, Borrow R, Oliver J, Rodrigues F, Morales-Aza B, Finn A. Whole genome analysis of longitudinal pharyngeal samples shows persistence of carriage with the same meningococcal strain".

As first author, I have given oral presentations of this work at international conferences: at the 2019 European Society for Paediatric Infectious Diseases Conference in Ljubljana, Slovenia; at the 2018 International Pathogenic Neisseria Conference in Asilomar, California; and at the 2017 European Society for Paediatric Infectious Diseases Conference in Madrid, Spain.

I have presented posters on this work as first author at the 2019 European Society for Paediatric Infectious Diseases Conference in Ljubljana, Slovenia; at the 2017 European Meningococcal and Haemophilus Disease Society in Prague, Czech Republic; and at the 2016 International Pathogenic Neisseria Conference in Manchester, UK.

In 2017, I was awarded the prize for best poster for “Tekletsion YK, Christensen H, Finn A. Direct multiplexed digital detection of *Neisseria meningitidis* genes using the NanoString nCounter system” at the Infection and Immunity Early Career Researchers’ Event and Vaccinology Symposium in Bristol, UK.

1.4 External contributors

The work described in this thesis was done by me, with the following exceptions:

- Pharyngeal swab collection for the carriage studies was done by the University of Bristol, Bristol Children Vaccine Centre for the BrisMenNHC and SPIT studies, and by Professor Fernanda Rodrigues’ research group, Coimbra Children’s Hospital for the Portuguese study.
- *sodC* quantitative polymerase chain reaction (qPCR) to identify *Neisseria meningitidis* from pharyngeal swab samples and measurement of bacterial carriage density was done by the University of Bristol, Finn laboratory group.

- Dr Caroline Cayrou did reverse transcriptase qPCR (RT-qPCR) for *fhbP* at the University of Leicester, I was observing.
- WGS of Nm isolates was done by Public Health England (PHE) (Manchester and Colindale). The sequences were uploaded into pubMLST by the University of Oxford.

Chapter 2. Background

2.1 The meningococcus

Nm, a Gram-negative diplococcus, also referred to as the meningococcus, is an obligate human pathogen and a normal commensal of the human pharynx. Carriage is a phenomenon in which bacteria asymptomatically colonise the body. Other important pathogens that colonise the upper respiratory tract are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* (2, 3). Carriage prevalence of Nm is age-specific in that it increases through childhood to peak in teenage years and then slowly declines during adulthood (4, 5). Around 10% of the world population are asymptomatic carriers of Nm. Exchange of respiratory droplets and secretions through close contact is the main route of transmission. Risk of carriage increases with social activity, intimate kissing and smoking (6) and in crowded conditions such as military barracks and pilgrimage sites e.g. the Hajj and Umrah (7-9). Meningococci can, on occasion, pass though the pharyngeal mucosa into the bloodstream to cause IMD, predominantly meningitis and septicaemia. IMD has a high case fatality ratio of around 10-15% (10, 11), with young children and teenagers being at highest risk of disease in most endemic settings (12). Other risk factors for IMD include close contact with a case of IMD, overcrowding, passive smoking and upper respiratory tract infections (13, 14). Certain medical conditions also increase the risk of invasive disease such as asplenia, complement deficiency and immunosuppression (15, 16). 10-20% of IMD survivors have serious long term sequelae such as neurological impairment, hearing impairment and limb amputation (17, 18).

The first recorded outbreak of IMD occurred in Geneva in 1805 as described by Vieusseux. Since then, outbreaks and epidemics have been documented around the world, although an area of sub-Saharan Africa stretching from Senegal in the west to Ethiopia in the east, known as the meningitis belt, is at particularly high risk of IMD every year with massive epidemics every 8-12 years (19). The bacterium was first identified by Weichselbaum in 1887 and Nm is now classified into 12 capsular groups based on the composition of the polysaccharide capsule (20). The large majority of disease is caused by six serogroups (A, B, C, W, X and Y). The predominant disease-causing serogroups vary over time and by region across the world (Figure 2.1).

Serogroups C, W, and X are currently the main epidemic disease-causing serogroups in sub-Saharan Africa, with MenB being the predominant cause of endemic disease across the Americas, Australia and Europe including the UK (21, 22).

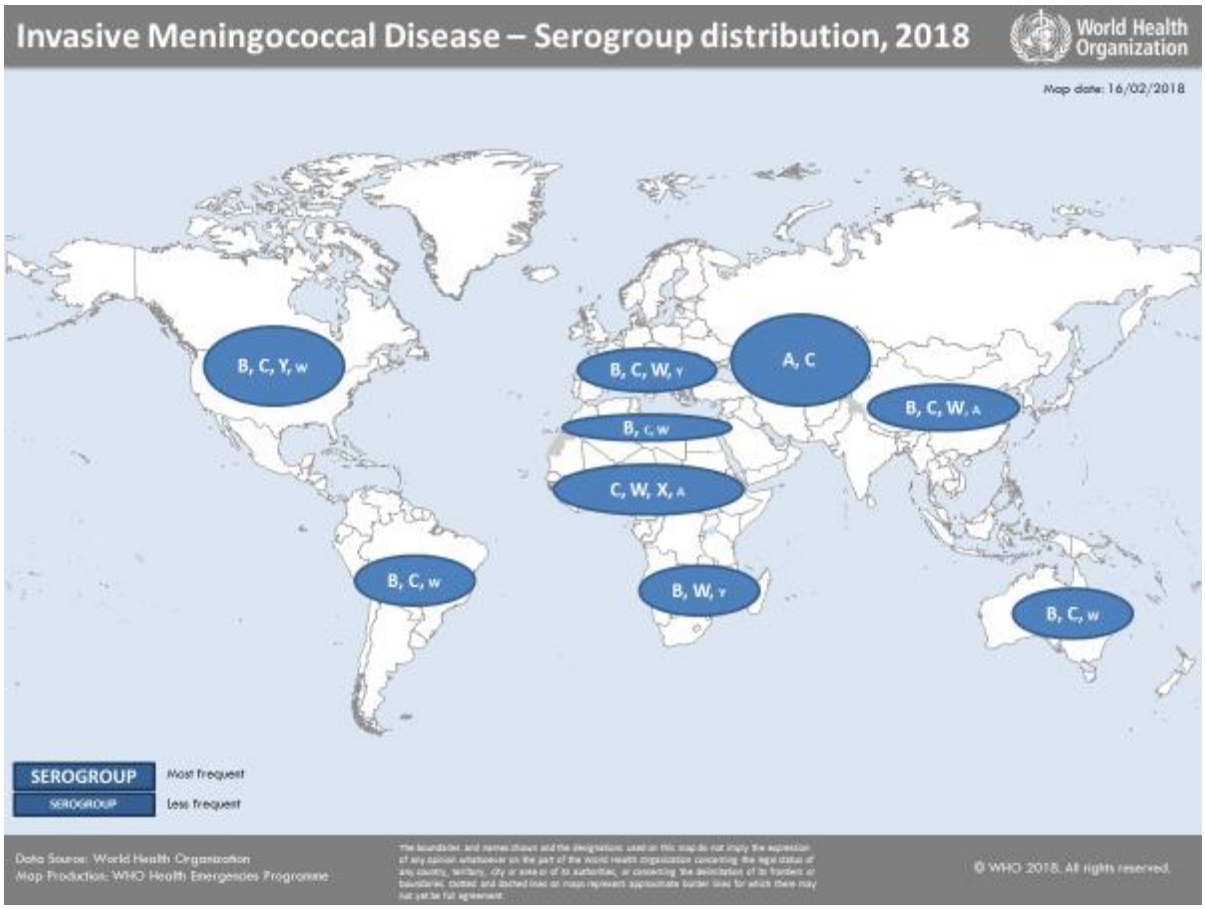


Figure 2.1 The distribution of Neisseria meningitidis major serogroups across the globe.

(Source:https://www.who.int/emergencies/diseases/meningitis/serogroup-distribution-2018.pdf?ua=1)

2.2 Nm genome

Nm strain MC58 (Genbank accession number AE002098), a pathogenic MenB strain, was the first neisserial strain to be gene sequenced in 2000 (23). This was also the first time that researchers used a genome annotation approach to identify the encoding genes of Nm in parallel with open reading frame (ORF) prediction. MC58 was found to have a genome length of 2,272,351 base pairs and about 2158 coding regions

(Figure 2.2) (23). Of these regions, 1158 were assigned a biological role with 85 predicted to be located on the surface of the bacterium (23).

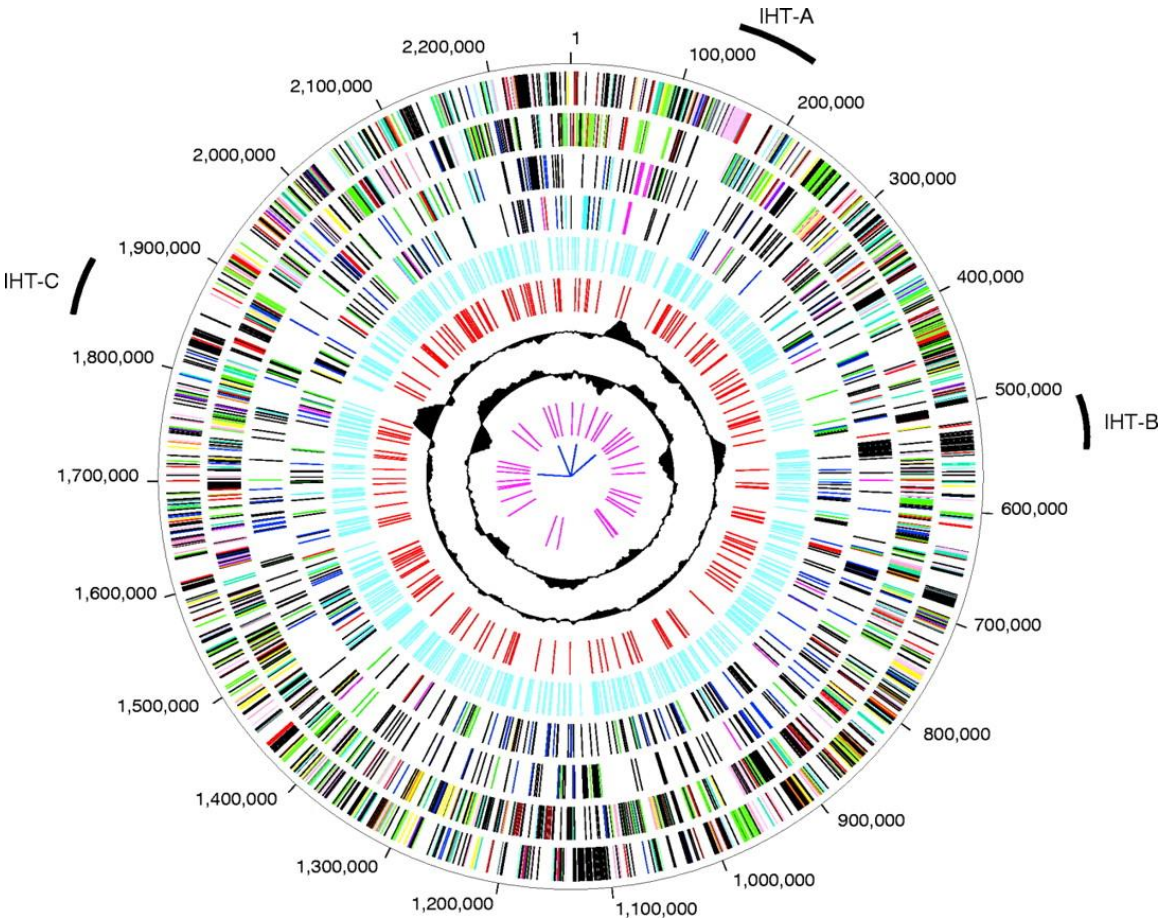


Figure 2.2 Circular representation of the Neisseria meningitidis strain MC58 genome (23).

2.3 Meningococcal vaccines

Prevention of meningococcal disease relies mainly on vaccination. Polysaccharide vaccines followed by improved polysaccharide-protein conjugate vaccines have been successfully developed against serogroups A, C, W, and Y. The search for an effective vaccine against MenB, the predominant disease-causing serogroup in Europe, has not been straightforward. Making a vaccine against MenB is challenging because of the high variability of surface-expressed proteins and the identical

chemical structure of the MenB capsular polysaccharide [α(2→8)N-acetyl neuraminic acid or polysialic acid] to glycosylation moieties found in human neural tissue which, being a self-antigen, is a poor immunogen (24, 25) .

2.4 Reverse vaccinology and MenB vaccines

The ability to sequence the full genome of the pathogen and the subsequent use of modern bioinformatics tools, a series of steps dubbed “reverse vaccinology”, revolutionized the traditional approach to vaccine design and permitted progress in developing MenB vaccines (23, 26).

Until recently the development of a vaccine followed the same techniques developed by Pasteur that involved isolating, purifying, inactivating or attenuating and administering the whole or part of the organism. Reverse vaccinology is a systematic search of genomic information to identify putative vaccine candidates in the protein-coding genome of a pathogen using bioinformatics, molecular biology, and immunology to select and synthesise suitable antigens. The process involves the following steps (i) bioinformatics software to screen the genome for surface expressed proteins (ii) high throughput expression of those proteins (iii) in-vitro confirmation of

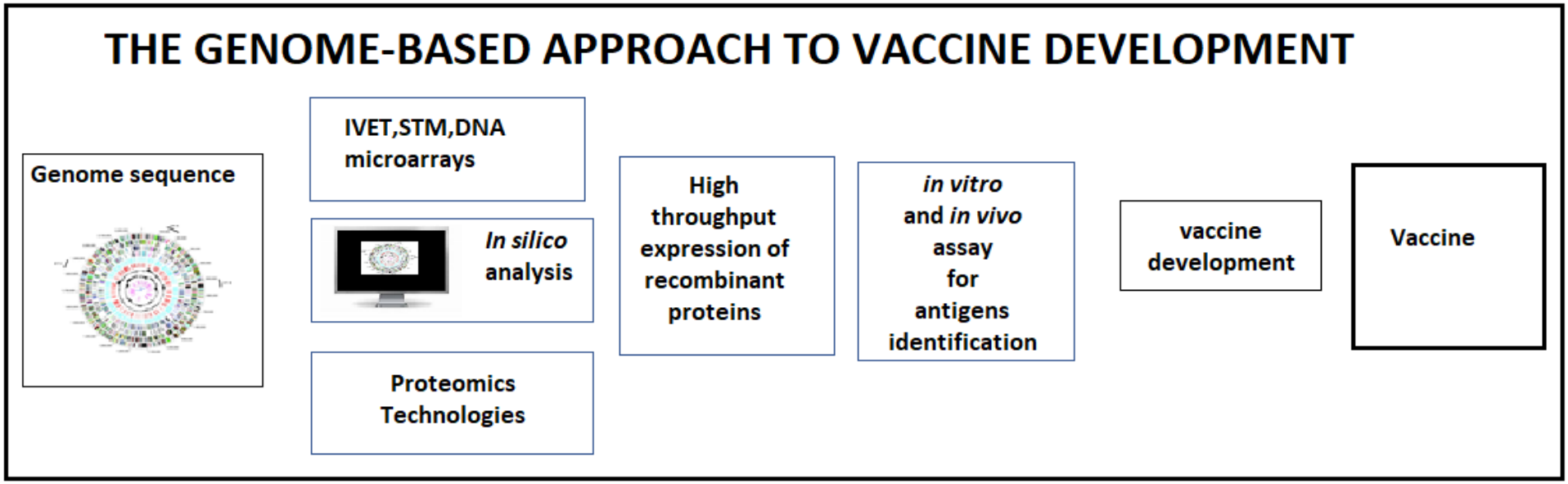


Figure 2.3 The genomic- based approach to vaccine development showing the steps of reverse vaccinology in the search for vaccine candidates, expression and purification, vaccination of suitable animal models and human clinical trials (27)

surface location (iv) animal-based immunogenicity testing, and finally (v) conventional vaccine trials (27) (Figure 2.3).

Using reverse vaccinology, Nm genome sequencing led to the development of a novel vaccine, 4CMenB (28) now licensed as Bexsero, the first vaccine candidate to be developed in this way (23). Once the sequence was obtained*, in-silico* antigen prediction from computer algorithms was used to predict and interpret the genome functions and to select novel virulence factors among surface exposed antigens. Among 570 Open Reading Frames predicted to encode surface expressed proteins, 350 were successfully expressed in *Escherichia coli* and used to immunise mice (23). Seven proteins that generated bactericidal activity were selected as potential vaccine candidates, tested for conservation in 22 representative MenB strains and 9 other neisseria species, and checked for antibody binding in the presence of capsule. Most proteins were conserved, and all were surface expressed. After further refinement and testing against a broader range of meningococcal strains, three main antigens (fHbp, NadA, NHBA) were selected for inclusion in the vaccine together with an outer membrane vesicle derived porA NZ98/254 meningococcal strain (28).

Bexsero was added to the UK universal primary vaccination schedule in September 2015 (29). Another similar protein vaccine (Trumenba) containing two fHbp variants has been licensed in the USA (30) and Europe. Research on the effectiveness and duration of protection of the two licenced MenB protein vaccines is ongoing. Preliminary data on impact of Bexsero suggests a vaccine effectiveness of around 80% against all MenB cases, with a 50% decline in MenB cases among those targeted for vaccination (31-33).

2.5 Mucosal immunity against meningococci

The mucosal layer is tolerant to most of the antigens encountered, including food, inhaled antigens, etc. and plays a major role when encountering an invading pathogen. Immune cells such as dendritic cells, which reside under the lamina propria, transfer antigens to secondary lymphoid organs that induce T cell activation (Figure 2.4). The T cells play a major role in regulating function by activating plasma cells and producing IgA antibody.

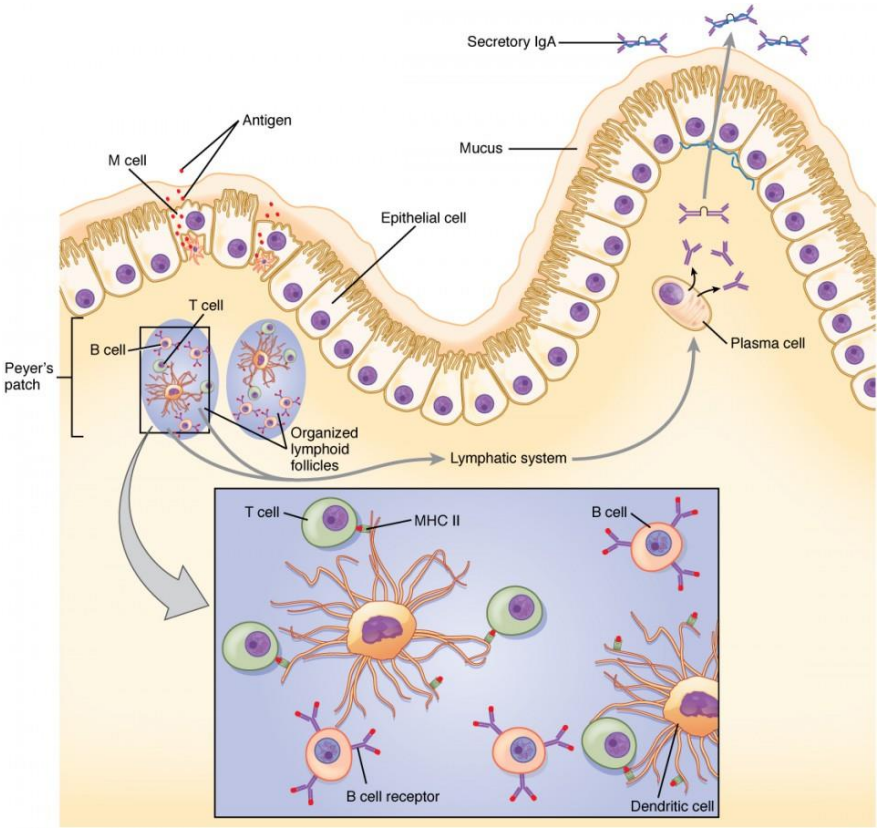


Figure 2.4 Representation of mucosal immune system

(Source:https://courses.lumenlearning.com/suny-ap2/chapter/the-immune-response-against-pathogens/)

Pathogens are recognised via many mechanisms such as by Pattern recognition molecular pattern molecules (PAMPs and toll like receptors (TLR4) that recognise, for

example, the Gram-negative surface lipopolysaccharide molecules (34). Macrophages are also important in killing the pathogen and inducing activation of the adaptive immune system through the major histocompatibility (MHC) complex (34). Macrophages are particularly important in recognition and killing of encapsulated Nm since the capsule makes peptide recognition sites relatively inaccessible (35). Neutrophils, T cells, B cells and other cells of the adaptive immune system are important in the protection against IMD (36, 37).

There is a high prevalence of meningococcal carriage compared to the incidence of disease. This is probably due to a number of factors such as natural mucosal immunity through secretory IgA and IgG antibodies (38-40), cellular immune response (41), innate immunity and the low virulence of many carrier strains (42). There is good evidence that colonisation by meningococci can elicit naturally-acquired immunity (43). Studies have shown that bactericidal antibody titres among meningococcal carriers are much higher than in non-carriers and that acquisition of Nm is followed within 1-2 weeks by an increase in antibody titres (44). There is also a hypothesis that cross-reactive immunity could be acquired through carriage of other neisserial species such as *N. lactamica (45-47)*. The latest studies suggest that carriage of *N. lactamica* inhibits acquisition of meningococci but may not generate protective immunity against IMD (48)

IgG antibodies are thought to be important in protection against IMD (49). It has also been shown that the T cell response to colonisation of meningococci is vital in modulating the protection by antibody class switching, affinity maturation and immunological memory (50). Goldschneider and colleagues demonstrated the natural protection induced by Nm against a specific serogroup C strain in long-term carriers by assessing serum bactericidal titres in those individuals (50). They also established

that innate immunity has an important role in protection against meningococcal infection. Recent research has suggested that the mucosal layer of the nasopharynx plays an essential part in protecting against invasion by the meningococcus and other commensals or pathogenic organisms due to specialised immune cells B cells which produce IgA. Such cells are known to reside in the lamina propria of the human epithelial barrier (51). There is also evidence of naturally acquired T cell-mediated mucosal immunity to Nm (52).

A recent study done in Togo to assess the natural capsular natural immunity against serogroup X meningococci suggested that the natural antibody titres were high and that the SBA against serogroup X indicated successful protection (53). Colonisation is known to induce serogroup specific antibodies and raised serum bactericidal antibody titres (54)

2.6 Effect of conjugate vaccines on carriage of Nm

Polysaccharide vaccines and protein-polysaccharide conjugate vaccines differ in their effectiveness. Polysaccharide vaccines are T cell-independent antigens that produce a reduced immune response in infants and give direct protection to the vaccinated individual for a limited period, whereas conjugate vaccines by combining with a protein antigen become T cell-dependent that leads to both long term and indirect population-wide protection (herd protection) (55). Differences in indirect protection between the vaccines can be explained by their effect on carriage. Polysaccharide vaccines possibly have an effect on carriage of Nm, but this is uncertain and any effect only lasts a few months (56). In contrast, protein-polysaccharide conjugate vaccines

appear highly effective at reducing carriage in the longer term (55). This means that carriage and transmission of organisms possessing the target polysaccharide antigens are greatly reduced. Vaccinating groups at high risk of carriage can therefore reduce the risk of disease, not only in the vaccinated but also in unvaccinated groups who are then less likely to be exposed to the disease-causing strains.

Vaccination with both pneumococcal and meningococcal conjugate vaccines leads to a decline in disease incidence due to vaccine capsular types/groups among the vaccinated population (54, 57) as well as in the unvaccinated age groups, well-illustrated by the effects of introduction of the meningococcal serogroup C conjugate vaccine (2, 58). Recent experience with a meningococcal serogroup A conjugate vaccine in the African meningitis belt has demonstrated a similar effect (59, 60).

2.7 Effect of MenB protein vaccines on carriage

The current recommendation is to offer Bexsero to UK children aged 2 and 4 months with a booster at 12 months. If Bexsero is effective in reducing prevalence of meningococcal carriage, then an alternative strategy for vaccine use could be immunisation of teenagers in the expectation of herd protection (5). One study suggests that Bexsero may reduce overall carriage of meningococci by around 18% (95%CI 3-30%) in the 3-12 month period after vaccination (61), but this estimate is uncertain and the duration of any such effect is unknown. Provisional results from a recent trial in Australia showed no evidence of a reduction in carriage of MenB strains after Bexsero (Marshall H, unpublished data).

2.8 Nm carriage density

It is well established that understanding the dynamics of bacterial carriage is important in the context of vaccination. For example, for the pneumococcus, the density and duration of bacterial carriage could have an effect on transmission and invasion by the bacteria (62). There is evidence that high pneumococcal carriage density is associated with mucosal inflammation (63). The density of nasopharyngeal carriage of *Streptococcus pneumoniae* in young children shows a normal distribution on a log scale peaking at 1,000-10,000 colony forming units/ml (Figure 2.5) (64). In contrast, the frequency distribution of Nm density among teenage carriers is heavily skewed to the left with the large majority having small numbers of detectable bacteria (Figure 2.6) (65).

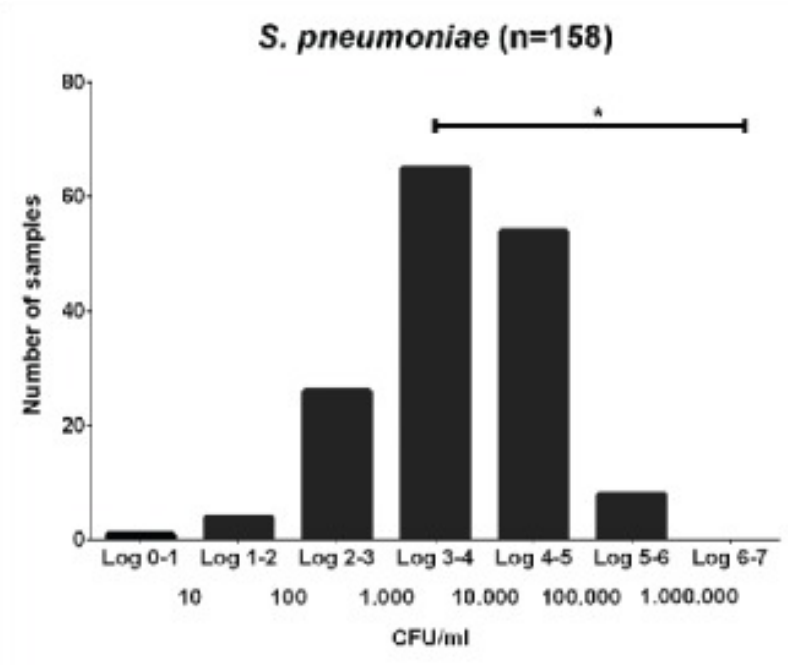


Figure 2.5 Distribution of bacterial carriage density of Streptococcus pneumoniae in carriage samples (61)

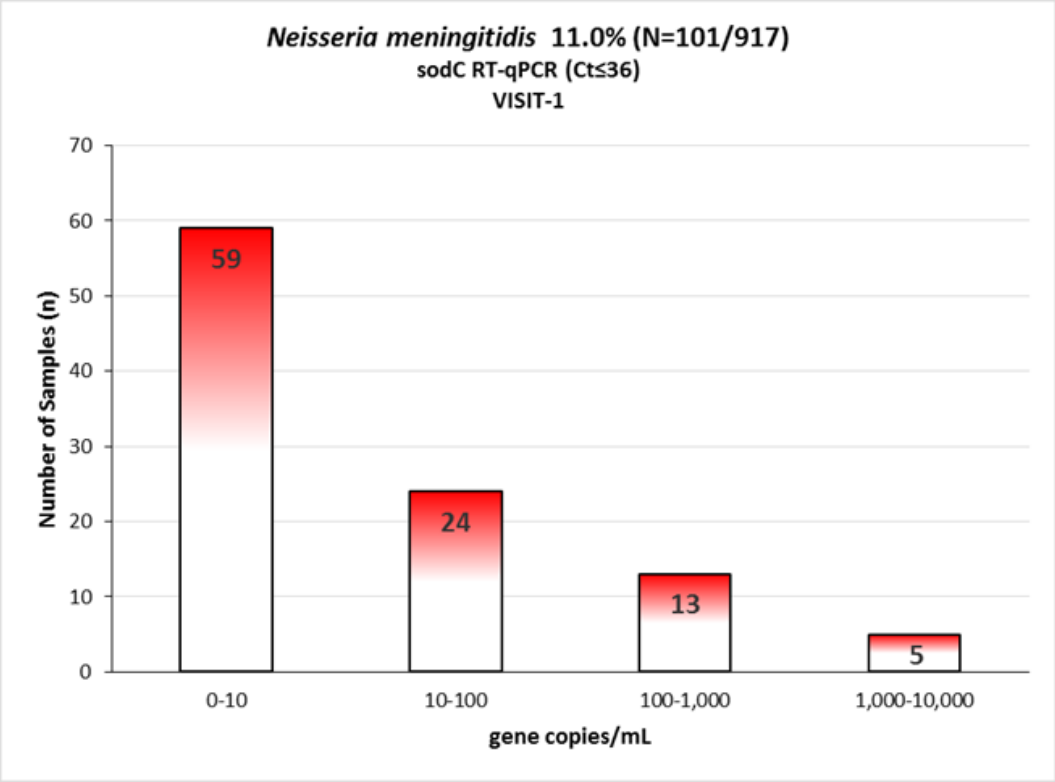


Figure 2.6 Bacterial carriage density of Neisseria meningitidis carriage samples from Bristol teenagers showing the distribution of carriage (66)

2.9 Interaction between Nm and humans

The interaction between meningococci with humans has been studied with techniques such as organ culture, primary cell culture, and immortalised cell lines, as there is no suitable animal model to study the mechanisms of attachment of and invasion by Nm (67). Different stages of the bacterial life-cycle have been studied using models to map progress from attachment to epithelial cells to invading the blood stream, attaching to endothelial cells, and eventually reaching the cerebrospinal fluid leading to meningitis (68). One of the mechanisms of immune evasion by Nm is by modulation of the surface expression of genes (69, 70).

Transcriptomic studies of Nm gene expression in pharyngeal carriage samples, its natural habitat, is critical to the study of the biology of the organism, including

establishment of colonisation, transmission and the occurrence of invasion and disease.

2.10 Transcriptomics

Transcriptomics describes the techniques used to study a complete set of transcripts or ribonucleic acids (RNAs) encoded in a genome of an organism. The main types of RNA include messenger RNA (mRNA), transfer RNA, and ribosomal RNA, which are involved in the transcriptional and translational processing of messages encoded in the genome of an organism (71). All cells in a multicellular organism contain the same deoxyribonucleic acid (DNA), but different cell types express different genes depending on the nutrient availability, environmental variability and other internal and external signals. The study of transcriptomes, or transcriptomics, also referred to as gene expression profiling, examines the expression level of mRNA in a given cell population at a given time (71).

If a gene carries information for protein synthesis, mRNA carries this information to a ribosome so that it can be translated into a specific protein. mRNA is the most unstable form of RNA with a half time of degradation in *Escherichia coli* of 1-3 minutes. This RNA degradation is important in the gene regulation process, but this instability makes the study of mRNA transcripts challenging.

2.11 Transcription in bacteria

Transcription is a multi-stage process of RNA synthesis from a DNA template involving the enzyme RNA polymerase (RNAP), a complex multi-subunit consisting of α2ββ’ϚϽ

subunits originally characterised by Burgess RR in 1977 (72). In bacteria, transcription and translation occur simultaneously in the cytoplasm.

Transcription occurs in three major steps after recognition of a specific transcription site (i) chain initiation, (ii) chain elongation and (iii) chain termination.

(i) Initiation. RNAP triggers initiation of transcription by binding to the transcription start site known as the promoter region of the DNA template (73). This is where most regulation of gene expression occurs by accessing or blocking this site to the RNAP. The promoter sequences are situated 10bp and 35bp upstream of the transcription start site (-10 and -35 sequence respectively). In bacteria, specific target recognition of the promoter site is guided by the sigma (σ) factor, a protein important for specific recognition of the bacterial promoter site. This σ factor forms a complex with the RNAP to open and unwind the double helix of DNA to initiate transcription (Figure 2.7)The complex of the sigma factor with RNAP is collectively called a holoenzyme (74-76).

(ii) Elongation is the second step of a transcription reaction. RNA synthesis proceeds from 5’ to 3’ direction catalysed by RNAP in the formation of phosphodiester bond between the ribonucleotides using DNA as a template (73, 75, 77-79). The RNAP links the nucleotides to the 3’ growing end of the mRNA molecule. Once a short RNA sequence starts to synthesize, the σ factor dissociates and RNAP elongates the growing transcript by moving from the promoter site to a full length of mRNA until the chain terminates.

(iii) Termination is the last step of a transcription reaction before protein synthesis. Once the RNAP reaches a terminator site, the RNAP, the DNA strand and the mRNA transcript dissociate from each other upon receiving a termination signal (protein or RNA based) (74, 80), and the transcribed RNA in this form (mRNA) carries information

for protein synthesis. It is at this point that the amount of transcribed mRNA in a bacterial cell can potentially be detected and measured by gene expression profiling.

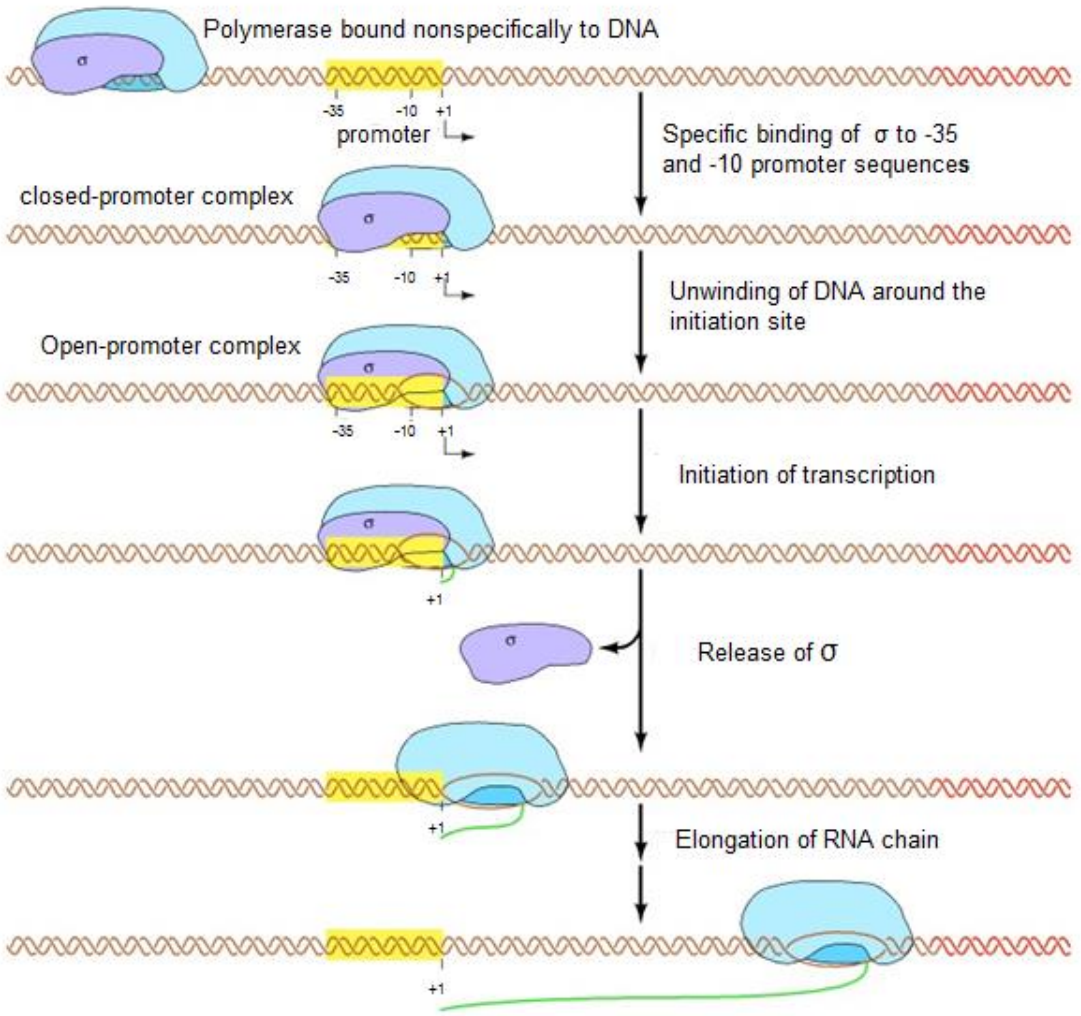


Figure 2.7 The three stages of bacterial DNA transcription process A) Initiation, B) Elongation, C) Termination Transcriptomics in the medical field (81)

Transcriptomic studies have played a major role in clinical diagnosis and research fields in recent years (82). They generally assess and identify genes that are differentially expressed and regulated in different tissues and cellular conditions, for

example, in the diagnosis of different bacterial and systemic diseases (83-86) and in biomarker identification of cardiovascular diseases and cancer. By profiling gene expression it is possible to monitor progress of disease and to design personalised treatments (87). Most importantly transcriptomic profiling is used to study immune responses induced by vaccines (88, 89), to assess potential vaccine antigens (90), to study response to environmental changes (1), and to identify candidates for new drugs and vaccines (91).

2.12 Nm transcriptomic studies

Transcriptomics are important in studying the dynamics of colonisation and invasion of the host epithelial cell by Nm (92). Nm has evolved a mechanism to escape the host immune system and survive adverse host micro-environments (e.g. iron starvation, heat shock, oxidative stress, carbon source limitation, lack of glucose), by expressing different genes or up/down regulating virulence genes (93, 94). Studies of the transcriptional responses to the micro-environment of the host can give a detailed picture of how the bacteria respond to different environment and nutrient changes. Transcriptomic analysis of *in vivo* pharyngeal samples could lead to understanding whether the new vaccines might interfere with colonisation and may enable us to predict vaccine efficacy indirectly. It may also lead to discovery of novel vaccine candidates with a potential impact upon acquisition and clearance of Nm. Similarly, the potential of specific mucosal immune responses induced by vaccines to impact upon acquisition or clearance of carriage is likely to depend upon levels of expression of the vaccine antigens by the bacteria at different densities and stages of colonisation.

Transcriptomic studies for Nm to date have used *ex vivo* models involving laboratory culture and cell lines to study the biology of the organism in the process of colonisation

and invasion of the host. *Ex vivo* studies have been done to assess the adaptation and survival of the bacteria in blood (95) or using cell lines to assess changes in gene expression when Nm attaches to epithelial or endothelial cells (92, 93) and during infection (93). Studies have also tried to characterise genes based on the transcriptomic profile of Nm using cultured isolates (96). There have been no previous transcriptomic studies of gene expression of Nm directly from *in vivo* human pharyngeal swab carriage samples.

For most transcriptomic work, RNA isolation and purification are necessary. Extracting RNA can be challenging especially for samples collected from a non-sterile site such as the pharynx that harbours many different bacterial species as well as human cells and debris. To this end, evaluating the efficiency of different RNA isolation techniques will be important for analysis of Nm gene expression.

2.13 Gene expression profiling

Gene expression profiling is a tool used to quantify the transcription of genes by measuring directly, or indirectly, the quantities and distribution of mRNA transcripts present in a particular cell preparation or tissue under specific circumstances, in order to provide a global picture of this aspect of cellular function. Gene expression levels vary greatly depending on the response to environmental conditions and the physiological state of a particular cell (97, 98), although certain housekeeping genes are consistently expressed and are important for the basic cellular functions of bacteria. Expression of such genes can be a useful yardstick against which to compare variations in expression of other genes whose transcription rates are less immutable.

2.13.1 Methods of gene profiling

Methods of gene expression profiling include sequencing techniques, relative gene identification or absolute transcript counting. Among the available methods, quantitative reverse transcriptase polymerase chain reaction (RT-qPCR), microarray and RNA sequencing (RNA-Seq) are the most commonly used.

RT-qPCR

RT-qPCR is one of the oldest transcriptomic techniques and it uses the reverse transcriptase enzyme to reverse transcribe RNA to complementary DNA (cDNA) to be amplified and detected using quantitative PCR (qPCR). This technique still is the method of choice of some researchers.

Microarray

Microarray uses short nucleotide probes that are attached to a solid substrate (a glass slide or silicon thin-film cell). The transcript abundance is determined by measuring the amount of fluorescently-labelled transcripts bound to these probes (99). The technique needs a prior knowledge of the genes and organism of interest to do the analysis, and measuring abundance using this method does not give an absolute gene count. The technique also needs reverse transcription of the RNA to cDNA, fragmentation and fluorescence labelling steps which could introduce bias.

RNA-Seq

RNA-Seq refers to quantification and detection of RNA (mRNA) by sequences generated from many short fragments of transcripts and uses computational methods to reconstruct the reads to a reference genome (100). RNA-Seq can identify genes which are active at a particular time in a particular tissue without prior knowledge of

the genes (100). Fragmentation of the RNA is a key step to get a reliable RNA-Seq result as mRNA is longer than the read length of the sequence. This technique requires fragmentation, library preparation, reverse transcription and alignment of the sequences.(101)

NanoString nCounter technology

NanoString nCounter technology is a recent advance in gene expression profiling techniques with fully automated gene expression detection and quantification methods (102, 103). The technology is based on digital detection and direct molecular barcoding of target transcripts using colour coded probe pairs. Each probe pair consists of a 50 base reporter probe, which carries the signal on its 5' end, and a 50 pair capture probe which carries a biotin on the 3' end. The reporter probe carries the signal; the capture probe allows the complex to be immobilized for data collection. The direct digital detection of mRNA using colour coded molecular barcoding avoids the need to reverse transcribe mRNA to cDNA and the need to amplify the gene. This method has been shown to give highly reproducible and accurate multiplexed results in a wide variety of samples (103).

Among the challenges in detecting RNA transcripts is that the quantity of RNA available may be insufficient to construct a RNA-Seq library or to label microarray analysis (104). Other techniques such as RT-qPCR need reverse transcription and amplification of RNA which can introduce bias into the analysis. Direct transcript detection of mRNA from a sample is an ideal way to get the exact picture in the cellular sample. The NanoString nCounter system has been shown to be effective in detecting mRNA from a sample without the need to amplify the gene, reverse transcribe or prepare a library file. This technique performs a direct transcript detection of multiple

targets using probe pairs in a single reaction and so is well suited to analysis of samples with low levels of transcript. The prerequisite for the NanoString system is a prior knowledge of the target genes of interest.

NanoString nCounter analysis has been shown to have a higher accuracy and reproducibility than techniques such as microarray and RT-qPCR and has an equivalent detection rate to RNA-Seq (105, 106). RNA-Seq generates large amounts of data that can be challenging for interpretation and library preparation for a small quantity of RNA (107). The other gene expression methods either involve conversion of the transcript message to cDNA or involve enzymatic reactions, which could introduce bias (107).

2.14 Summary

Transcription studies of samples from the human upper respiratory tract of genes that code for the meningococcal proteins in the two licensed MenB vaccines and other potential protein vaccine antigen candidates may provide information about their potential to induce protective mucosal immune responses. The NanoString system offers a potential method to study gene expression of Nm from *in vivo* pharyngeal samples that has not been previously used. If certain genes are expressed more in pharyngeal carriage, this could advance our understanding of the colonisation process and the potential protection from protein vaccines.

2.15 Aim and objectives

2.15.1 Aim:

To describe bacterial transcription signatures of Nm colonising the human pharynx (and at different densities) in order to evaluate whether gene products either in current vaccines, or other potential vaccine candidates, might be involved in acquisition, colonisation and transmission of meningococci, and thereby their potential effectiveness in reducing carriage.

2.15.2 Objectives:

- To establish methodology for extraction and quantification of RNA from cultured Nm isolates and from pharyngeal swabs obtained from meningococcal carriers.
- To select a panel of meningococcal genes encoding proteins in the current MenB vaccines, those under consideration as vaccine candidates, and others that may have roles in colonisation and response to stress.
- To establish mRNA transcript detection assays (NanoString) for the selected panel of genes
- To compare the gene expression profiles of meningococci cultured under different environmental conditions, thus confirming the potential to detect changes in expression.
- To establish gene expression from *in vivo* human pharyngeal samples and compare gene expression profiles in high and lower density carriers
- To examine whether gene expression varies with density, strain, social and demographic factors.

Chapter 3. Materials and Methods

3.1 Materials

3.1.1 Reagents

Reagents	Supplier
Acid: phenol: chloroform: isoamyl alcohol	Thermo Fisher Scientific, UK
RNaseZap solution	Thermo Fisher Scientific, UK
Tris-EDTA buffer	Sigma-Aldrich Co. Ltd, UK
Absolute ethanol	Sigma-Aldrich Co. Ltd, UK
Sodium acetate	Sigma-Aldrich Co. Ltd, UK
Lysozyme	Sigma-Aldrich Co. Ltd, UK
Isovitalex enrichment	Becton Dickinson Ltd, UK
Phosphate buffered saline with Mg and Ca	P.A.A. Laboratories Ltd, UK
RNase free Phosphate buffered saline	Thermo Fisher Scientific, UK
2-Mercaptoethanol	Sigma-Aldrich Co. Ltd, UK
Deferoxamine mesylate salt	Sigma-Aldrich Co. Ltd, UK
Glacial acetic acid	Sigma-Aldrich Co. Ltd, UK
Horse blood	Oxoid Ltd, UK

Reagents	Supplier
Proteinase K	Qiagen Ltd, UK
Nuclease free water	Qiagen Ltd, UK
RNAlater	Thermo Fisher Scientific, UK
RNAprotect	Qiagen Ltd, UK
Reporter probes	NanoString technologies, USA
Capture probes	NanoString technologies, USA
NanoString hybridisation buffer	NanoString technologies, USA
RNasin® Ribonuclease Inhibitor	Promega UK, UK
Tryptic Soya broth	Becton Dickinson Ltd, UK
Reagent A	NanoString technologies, USA
Reagent B	NanoString technologies, USA
Reagent C	NanoString technologies, USA
Hybridisation buffer	NanoString technologies, USA
Tag polymerase	Thermo Fisher Scientific, UK
Hot start (dNTPs)	Thermo Fisher Scientific, UK
DNase	Promega UK, UK
Reverse transcriptase	Thermo Fisher Scientific, UK
Fast SYBR green	Thermo Fisher Scientific, UK

Reagents	Supplier
JAMES reagent	Biomerieux Ltd, UK
ZYM B	Biomerieux Ltd, UK
NaCl	Biomerieux Ltd, UK
Mineral oil	Biomerieux Ltd, UK
Horse blood	TCS Biosciences Ltd, UK
Columbia agar with horse blood	Thermo Fisher Scientific, UK
G.C. Selective Agar with VCNT	E & O laboratories, UK
XT_GX CodeSet	NanoString technologies, USA
nCounter master kit	NanoString technologies, USA
BHI broth	Thermo Fisher Scientific, UK
BHI agar	Thermo Fisher Scientific, UK
Beta 2-Mercaptoethanol	Sigma-Aldrich Co. Ltd, UK

3.1.2 Materials

Material	Supplier
25ml universal tubes	University store
Vented cap tubes	Sigma-Aldrich Co. Ltd, UK
15ml and 50ml graduated yellow top tubes	Sigma-Aldrich Co. Ltd, UK
2ml RNase/DNase free microfuge tubes	STARLAB, UK
1.5 ml RNase/DNase free microfuge tubes	STARLAB, UK
RNase free 1000,200,20,10 tips	Thermo Fisher Scientific, UK
Spectrophotometer with cuvettes	Sigma-Aldrich Co. Ltd, UK
Spectrophotometer without stoppers	Sigma-Aldrich Co. Ltd, UK
2ml microcentrifuge Tubes	STARLAB, UK
Barrier (filter) tips	Thermo Fisher Scientific, UK
RNase DNase free 1.5ml microfuge tubes	STARLAB, UK
SPRINT Cartridge	NanoString technologies, USA
MAX Cartridge	NanoString technologies, USA
Ampule rack	Biomerieux Ltd, UK
Ampule protector	Biomerieux Ltd, UK

3.1.3 Commercial kits

Kits	Supplier
RNeasy mini kit	Qiagen Ltd, UK
RNeasy minElute Cleanup kit	Qiagen Ltd, UK
RNase free DNase set	Promega UK
nCounter master kit	NanoString technologies, USA
nCounter CodeSet Kit	NanoString technologies, USA
RNase protect bacteria mini kit	Qiagen Ltd, UK
nCounter gene expression CodeSet	NanoString technologies, USA
API_NH	Biomerieux Ltd, UK
NanoString Cartridge (SPRINT and MAX)	NanoString technologies, USA
NanoString plates	NanoString technologies, USA

3.1.4 Instrumentation

Instruments	Supplier
Spectrophotometer	Thermo Fisher Scientific, UK
Heating block	Grant Instruments, UK
Mini-microfuge (nano)	Thermo Fisher Scientific, UK
Microcentrifuges	Thermo Fisher Scientific, UK
Vortex	Scientific Industries, UK
Shaker	Stuart Scientific, UK
CO_2 Incubator	Thermo Fisher Scientific, UK
Microcentrifuge	Labnet, UK
NanoString nCounter pre-station	NanoString technologies, USA
NanoString nCounter digital analyser	NanoString technologies, USA
PCR thermal cycler	MJ Research Inc, USA
Denovix DS-11 spectrophotometer	DeNovix Inc, USA
Plate counter	Stuart Scientific, UK
Water bath	Grant Instruments, UK
Pipettes L10, L20, L200, L1000	Sartorius Ltd, UK
Rainin pipettes L10, L20, L200, L1000	Mettler-Toledo Ltd, UK
Tube-strip picofuge 200µl	Thermo Fisher Scientific, UK

Instruments	Supplier
200µl PCR tubes	STARLAB, UK
nCounter reagent plates	NanoString technologies, USA
NanoString Prep plates	NanoString technologies, USA
Racked tips for prep station	NanoString technologies, USA
NanoString SPRINT	NanoString technologies, USA
Mixing robot (QIAgility)	Qiagen Ltd, UK
qPCR machine (QIAsymphony)	Qiagen Ltd, UK

3.2 Methods

3.2.1 Sterilisation of equipment

All glassware was washed and sterilised at 160ºC for 3 hours to minimise RNases that could degrade RNA in the samples. 25ml screw capped universal tubes were soaked in 100% ethanol for 30 mins, rinsed three times with double distilled water and baked for 3 hours at 160ºC in a dry oven. All the sterilised materials were handled with protective gloves to avoid skin contact that could contaminate the glassware with RNases.

3.2.2 Nm cultures and growth media

3.2.2.1 Culture (Colombian blood agar)

American Type Culture Collection (ATCC)-BAA-335 MC58 Nm strains were cultured on Colombian blood agar (CBA) containing 5% horse blood at 37ºC for 16 hours in 5% carbon dioxide (CO_2). The bacteria were stocked in tryptic soy broth plus 15% glycerol and kept at -80ºC. All the subsequent experiments were performed from a fresh stock of Nm.

3.2.2.2 Suspension feeder culture

Single Nm colonies were taken from an overnight growth culture on CBA with a plastic loop and emulsified slowly into a tube containing 3ml phosphate buffered saline with calcium and magnesium (PBS-B) by rubbing the colonies onto the inside wall of the tube. After leaving the suspension to settle for 1 minute, 2ml was transferred to another sterile universal tube, avoiding aggregated bacteria at the bottom of the tube. The suspension was made to have an absorbance value OD_{600} = 1 for use as a feeder

culture to inoculate brain heart infusion (BHI) broth (enriched by 10% heat inactivated horse blood and 2% isovitalex) at 1:100 dilution.

3.2.2.3 Culture (GC agar)

GC selective culture media was used to isolate Nm from the BrisMenNHC pharyngeal swab samples (Appendix D). GC agar contains antibiotics which inhibit the growth of gram negative and positive bacteria and supports the growth of Nm. Samples stored in STGG at -80ºC were thawed on ice for 2 hours. 100ml of the defrosted sample was plated onto GC plates. The media was incubated for 24, 48 and 72 hours until a typical Nm colony was observed.

3.2.2.4 Liquid culture in GC broth

A broth GC culture media was prepared and enriched before adding the bacterial suspension. The media were enriched using 10% heat inactivated horse blood and 2% isovitalex. The GC broth was prepared to have 12.5µM Fe $(NO_3)_3.9H_2O$.

3.2.2.5 Liquid culture in BHI broth supplemented with 10% horse blood

BHI broth was used to grow Nm from a feeder Nm culture, enriched by 10% heat inactivated horse blood and 2% isovitalex. A suspension of Nm was made (3.2.2.2.), inoculated in 1:100 dilution to the broth and incubated at 37ºC in a 5% CO_2 incubator, shaking at 100rpm.

3.2.2.6 Heat inactivated horse blood

Horse blood was heat inactivated in the department laboratory. The blood in a sterile bottle was heated in a water bath set at 56ºC for 30 minutes, swirling the bottle every 10 minutes. The heat inactivated blood was cooled to room temperature and

dispensed into 15ml and 50ml sterile tubes. The tubes containing the heat inactivated blood were frozen at -20ºC until needed. The sterility of the heat inactivated blood was checked by culturing a drop of it on CBA overnight.

3.2.3 Pharyngeal swab collection, transport and storage

Informed consent was obtained from subjects prior to taking pharyngeal swab samples. The samples were taken using a double headed swab, sweeping from one tonsil, behind the uvula to the other tonsil. The two heads of the swab were snapped off and put into two different media. One head of the swab was stored in 1.5ml skim milk, tryptone, glucose, and glycerine (STGG) in a 2ml Eppendorf tube, and the other was kept in 1.5ml RNAlater. The tubes were stored at 2-8ºC during sampling and transported to the laboratory within 2-6 hours of collection. On arrival at the UoB laboratory, the STGG samples were vortexed for 30 seconds before storage at -80ºC, whereas the RNAlater samples were vortexed and stored at 4ºC overnight before being transferred to a -80ºC freezer.

3.2.4 Ethical clearance

Ethical approval to use the samples held in the Bristol Biobank (project ID: U-II-0023) was obtained for 267 Nm positive BrisMenNHC samples collected both in RNAlater and STGG. Analysis of swab samples from the SPIT study was approved by the South West - Frenchay Research Ethics Committee (REC reference: 16/SW/0269) and from the Portugal study was approved by the Ethics Committee of the Coimbra School of Medicine, Portugal (CE-6/2012). Subjects who did not give consent for further DNA analysis were excluded from the study.

3.2.5 RNA extraction from culture and pharyngeal swab samples

Prior to RNA extraction, 1.5ml RNAlater bacterial culture and pharyngeal swab samples were thawed on ice, diluted with an equal volume of ice cold 1x phosphate buffered saline (PBS) and centrifuged at 5000g for 2 minutes. The supernatant was discarded with careful pipetting. The pellets were re-suspended with 20µl RNase free water and treated with 15mg/ml lysozyme plus Tris-EDTA buffer in the presence of PK (proteinase K) to lyse the bacteria cells for 30 minutes at room temperature, shaking for 11 seconds every 2 minutes.

Bacterial RNA was extracted and purified using the RNeasy mini kit. Once the bacterial cells were lysed and homogenised, a 1:10 diluted 700µl pre-prepared mixture of beta 2-mercaptoethanol to RLT Qiagen buffer was added and vortexed vigorously. 2-mercaptoethanol prevents the degradation of RNA by nonspecific RNases. Absolute ethanol (500µl) was added and mixed to promote selective binding of RNA to the RNeasy membrane, then 700µl of the mixture was applied into the RNeasy mini-column at a time. The RNA binds to the column and other contaminants are washed through during micro-centrifugation for 15 seconds at 10,000rpm. 500µl RW1 wash buffer was added to remove biomolecules such as carbohydrates, proteins, fatty acids etc. Molecules with >200 bases remain bound to the column. On-column DNA digestion was performed for all the samples (3.2.6). A second wash was done using RW1 followed by 500µl RPE buffer, a mild wash buffer that removes traces of salt in the column. The column was centrifuged for 1 minute to dry the column and the RNA was eluted from the column by adding 30µl RNase-free water.

3.2.6 DNA digestion using Qiagen DNase kit (on column DNA digestion)

On-column DNase digestion was performed for all the samples using RNase free DNase set. DNase l stock solution was prepared by injecting 550µl RNase-free water into a DNase I vial using sterile needle and syringe. The DNase solution was mixed by gently inverting the vial and 50µl of the solution was aliquoted and kept at -20ºC. When needed, DNase solution was thawed at room temperature and gently mixed with 10ul DNase stock with 70µl of buffer RDD and centrifuged briefly. The DNase solution was added directly to the RNeasy column containing the sample and left at room temperature for 15 minutes. 350µl RW1 buffer was added to the RNeasy column to remove biomolecules such as digested DNAs, carbohydrates, proteins and fatty acids etc. that are non-specifically attached to the silica membrane.

3.2.7 DNA digestion using TURBO DNase (off column DNA digestion)

The total RNA samples extracted using RNeasy mini kit and stored at -80ºC were thawed on ice. After adding 10X TURBO DNase buffer to 1x concentration of the thawed RNA sample, 1µl TURBO DNase was added and incubated at 37ºC for one hour. Inactivation reagent was added to inactivate the TURBO DNase. Samples were kept at -80ºC until needed.

3.2.8 Isolation of Nm from STGG collected pharyngeal swab sample

Pharyngeal swab samples in STGG stored at -80ºC were thawed slowly on ice for 2 hours. The samples were vortexed for 30 seconds at high speed to homogenise the samples. 100ml samples were then plated out on GC selective media. The samples

were left to grow for 24 hours, 48 hours and 72 hours at 37ºC in a 5% CO_2 incubator before doing sub-culture. Typical Nm colonies were selected from the GC media, sub-cultured on CBA agar, and incubated at 37ºC for 24 hours in a 5% CO_2 incubator. Any media contaminated with bacteria other than Nm were further sub-cultured on CBA agar as above, whereas visually uncontaminated media went on for Gram-stain, oxidase test and API biochemical tests.

3.2.9 Bacterial lysate preparation

Bacterial cell lysate was prepared using lysozyme with TE and RLT buffers from Qiagen. The frozen Nm samples in RNAlater were defrosted on ice, added to an equal volume of ice-cold PBS and centrifuged for 2 minutes at 5000g. After treating the bacterial pellet for 30 minutes with 15% lysozyme and TE buffer in the presence of PK, RLT buffer was added before proceeding to hybridisation reaction and gene expression analysis.

3.2.10 Oxidase test

A single colony from an overnight culture on CBA was taken using a sterile plastic loop and emulsified onto a filter paper with 50µl oxidase reagent (tetraethyl_p_phenylenediamine dihydrochloride). The colour change when applying the bacteria was observed after 10 seconds and recorded. If the bacteria produce cytochrome oxidase, the oxidase reagent is hydrolysed to a purple colour (oxidase positive). If the paper remains colourless (white), this is interpreted as oxidase negative.

3.2.11 Gram stain

One colony was taken from the overnight grown Nm CBA plate and a thin smear was applied on a glass slide with a drop of (50µl) distilled water covering an area of approximately 2x3cm.The smear was air dried, fixed using a flame by passing the slide through the flame 2 to 3 times and left to cool for 1 minute at room temperature. The smear was covered with a primary stain, crystal violet, for 30 seconds and washed off gently with distilled water. Logol's iodine was added as a mordant for 30 seconds and washed off as above. Finally, the smear was covered with safranin, a secondary stain, for 30 seconds, washed and air dried before examining the morphology and Gram reaction of the bacteria under a microscope.

3.2.12 Biochemical test with API_NH

A bacterial suspension was made from samples identified as oxidase-positive Gram-negative diplococci to assess the biochemical behaviour of the bacteria to identify putative meningococci. The bacterial suspension was made in 0.85% NaCI (normal saline) from a purified growth of Nm on a CBA plate by selecting well-isolated colonies, with a MacFarlane strand of 4.0. The suspension was distributed into ten microtubes avoiding formation of air bubbles. 50µl suspension and 100µl mineral oil were added to each of the first 7 microtubes containing dehydrated substrate, and 150µl suspension was added to each of the last three microtubes. The tubes were incubated for 2.5 hours at 35ºC in aerobic conditions. The results were read visually by referring to the reading table provided by the company. The last three microtubes were read before adding reagent. A drop of ZYM B reagent was added to microtubes 8 (LIP/ProA) and 9 (PAL/GGT) and JAMES reagent was added into microtube 10(βGAL/IND). After

three minutes the result was read again. The results were read and interpreted using the table provided. A control ATCC strain was run alongside the test.

3.2.13 RNA Preservation media

3.2.13.1 RNAlater

RNAlater is a liquid RNA preservative solution that allows rapid stabilisation and protection of cellular RNA integrity. This solution was used to preserve RNA collected on swab samples, during transport to the laboratory within 2-6 hours of sample collection, and on pelleted Nm cultures in the laboratory.

3.2.14 Nucleic acid quantification and integrity testing

3.2.14.1 NanoDrop

Quality and quantity of the RNA extracted from carriage samples or cultures was measured using a Nanodrop Denovix D-11+ spectrophotometer. The quality of the samples was measured by taking the ratio of absorbance at 280nm to 260nm. A ratio of 1.9 to 2 was taken as sufficiently pure. The quantity of RNA was measured after blanking the spectrophotometer with 1µl of RNase DNase-free water. The same volume of total RNA was used to measure the absorbance of the samples.

3.2.14.2 RNAtape station

Total RNA samples, extracted using RNeasy mini kit from culture, were assessed using electrophoretic separation on micro-fabricated chips. RNA concentration and the

ribosomal ratio of 16S/23S, automatically generated from the electrophoretic bioanalyzer as a RIN number, were used to assess the integrity of the samples.

Quality and quantity of RNA was checked using the Agilent 2200 tape station system, an automated RNA screening technique using prepacked reagents. The assay also provides the ability to detect gDNA contamination of the total RNA samples. Fifteen samples from the iron/temperature stress experiments (chapter 4) were diluted to 100ng/µl from the stock RNA samples and analysed using Nanodrop and RNA screen tape analysis. The samples were aliquoted in 3µl to two 0.2ml PCR tubes. One was sent to the Department of Life Sciences, UoB, where sample quality was checked using RNA tape station and the other was assessed using Nanodrop. Contamination of the RNA samples with gDNA was assessed with the RNA screen tape assay.

3.2.15 NanoString nCounter instruments and techniques

3.2.15.1 Instruments

The NanoString nCounter system is a new and powerful technique of gene detection and counting developed by NanoString Technologies, Seattle, USA. The platform uses high resonance microscopic imaging and molecular barcoding of genes using specific colour coded probes. This system can be used to profile RNA, DNA and protein simultaneously in a single reaction, measuring up to 800 gene labels.

Two different types of nCounter profilers were used in these studies for assaying gene expression of Nm from culture and pharyngeal swab samples. The culture samples were assayed at University College London using the MAX/FLEX nCounter instrument whereas the *in vivo* pharyngeal swab samples were assayed in Bristol using the

SPRINT analyser. The MAX/Flex nCounter system comprises two instruments, a prep-station fluidic handling robot and a digital data analyser whereas the fully automated SPRINT profiler is a one-set digital analyser with an embedded prep station and digital analyser.

Below is the workflow of Nm gene expression from RNA extraction to obtaining mRNA to data analysis (Figure 3.1).

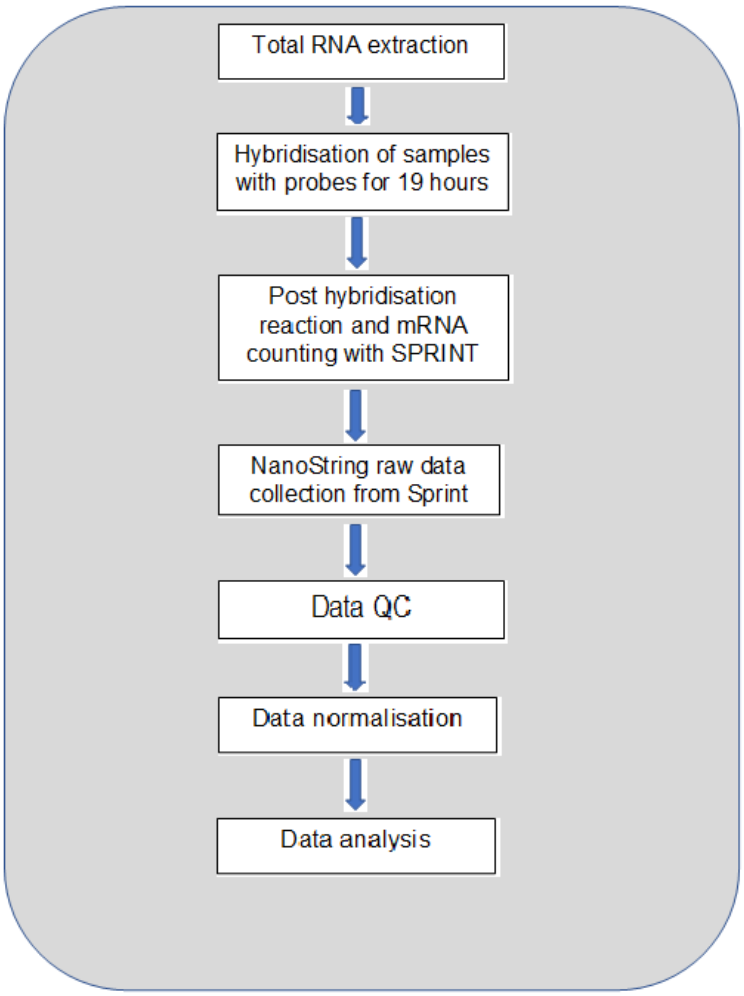


Figure 3.1 Gene expression profiling of Neisseria meningitidis workflow

3.2.15.2 Sample hybridisation

Hybridisation was performed according to the manufacturer's recommendations following the total RNA XT protocol (108). All reagents were brought to room temperature and the samples were thawed on ice. A master mix was prepared by adding 70µl hybridisation buffer to the tube containing the reporter probe set and mixed by tube inversion. After a quick spin of the master mix, 8µl were aliquoted into 12 pre-labelled 200µl PCR tubes. 5µl total RNA/lysate and 2µl capture probe set were added to each tube before placing the samples in a pre-heated thermocycler at 65ºC. The mixture was incubated in a heated lid thermocycler at 80ºC for 19 hours to let the probes hybridise to the target sequences (Figure 3.2).

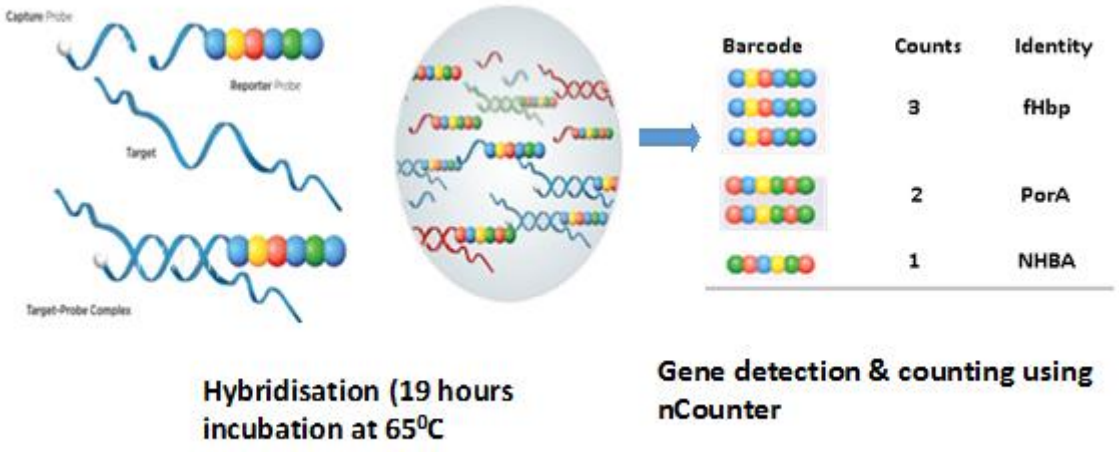


Figure 3.2 Schematic representation of steps involved in NanoString nCounter hybridisation and gene detection

3.2.15.3 Post hybridisation reaction

The overnight hybridised samples were taken out of the thermocycler after 19 hours incubation. 15µl RNase DNase free water was added to each tube to bring the final

volume to 30µl and loaded into a room temperature thawed NanoString cartridge. The cartridge was put in the SPRINT/MAX FLEX profiler for mRNA transcript counting. Each of the hybridised samples was loaded in the cartridge according to the manufacturer's protocol by pushing the pipette plunger to the second stop, creating an air bubble and removing the pipette before releasing the plunger to avoid sucking the sample out. The top of the cartridge was wiped with a clear dry wipe and the sample port sealed with a transparent seal. The removable green seal was removed from the cartridge before putting into the SPRINT analyser for gene detection and counting

The post hybridisation process for the MAX/FLEX requires loading the overnight hybridised samples onto a fluid handling prep station. All the ready-to-load reagents (the master kit) required for sample processing on the prep station were provided by NanoString with the CodeSets. These reagents and consumables were loaded onto the prep station prior to loading the hybridised samples onto the instrument. All the reagents were brought to room temperature before processing. The prep plates containing the magnetic beads were spun for 1 minute at 2000rpm to make sure that the beads were at the bottom of the well. Other materials such as the sample cartridge, empty strip tubes, tips, tip sheaths and tip wastes for liquid waste were placed in specified places on the instrument. The overnight hybridised samples were then placed on the sample port along with the reagents for two-step magnetic bead-based purification. Magnetic beads capsulated with a short sequence complementary to the capture probes and the reporter probes bound to the complementary sequences. Excess unhybridized probes and non-target cellular transcripts that are not attached to the cartridge were removed using the prep-station leaving the hybridised complexes attached. The process on the prep station took two and a half hours to process 12

samples. A cartridge containing the captured hybridised probes was transferred to the digital analyser for transcript detection and counting.

3.2.15.4 nCounter Digital data capturing and analysis

The reporter library file (RLF), generated by NanoString to contain information about the unique custom CodeSet was loaded onto the nCounter digital analyser. The data were captured from the cartridge by taking images of the immobilised florescent reporter probes with a charge coupled device (CCD) camera at the highest data resolution, 600 or 194 fields of view (FOVs) according to the machine used (Max/Flex or SPRINT), corresponding to the reporter code count (RCC). The data were exported as comma separated value (CSV) files using a memory stick for analysis.

3.2.15.5 Internal QC

For mRNA gene expression assays, 6 internal positive and 8 negative controls had been added to the CodeSet by NanoString Technologies during manufacturing to assess the overall technical performance of the assay. The positive and negative controls were synthetic oligonucleotides made to be non-homologous to any known organism and validated by the External RNA Control Consortium (ERCC). The six internal negative controls denoted alphabetically from A-H (NegA-NegH) were made to have no transcript to estimate the non-specific background signal, positive controls were pre-mixed with the reporter probe. The positive controls were spiked in at concentrations of 128, 32, 8, 2, 0.5 and 0.125fM when manufacturing the probe set.

The background count (“raw” data) were adjusted using negative control counts of the eight negative controls included with the CodeSet. The geometric mean of the eight negative controls were calculated for each lane and deducted from each raw count to

adjust the background count. Positive spiked controls were used to assess the quality and linearity of imaging. The normalisation was done on nSolver and the data were exported to Stata version 15 for further analysis (109).

3.2.15.6 Data QC

The data on raw gene counts for every gene assayed were extracted and data QC performed using four QC metrics (positive control linearity, imaging, binding density, and limit of detection). The QC was done following the manufacturer's manual (110).

(i) Positive control linearity

The positive control linearity was checked automatically using nSolver™ 3.0 analysis software using the positive controls included in each assay in different concentrations (3.2.15.5). As the lowest value positive control (*POS_F)* is considered below the limit of detection of the system, it was excluded from this calculation. The Pearson correlation coefficient is calculated from the regression between the known concentration of each of the positive controls and their actual counts. This measures the efficiency of the hybridisation reaction. A correlation of R^2 =0.95 was taken as the cut off value (Figure 3.3).

$$R^2 = \text{linear regression of } \frac{\log_2 \text{known (positive controls)}}{\log_2 \text{measured (positive controls)}} >= 0.95$$

Figure 3.3 Linear regression of positive controls calculation to check the linearity performance of the assay

(ii) Imaging QC

The image on a NanoString cartridge is taken in small subdivided sections/areas (FOVs) where the genes (barcodes) are scanned and counted (Figure 3.4). The sum of gene counts in FOVs from a single lane gave the final raw count for each gene and was reported as FOVs counted. The SPRINT analyser counts 194 FOVs and the MAX/FLEX analyser counts 600 FOVs as set by the NanoString company.

The imaging percentage is a ratio of the number of FOVs that have been successfully scanned (FOVs counted) divided by the total number of FOVs that the instrument makes an attempt to scan (FOV count). Percentages greater than 75% were taken as a good technical performance of the assay as recommended by the company. This QC was performed for each sample analysed.

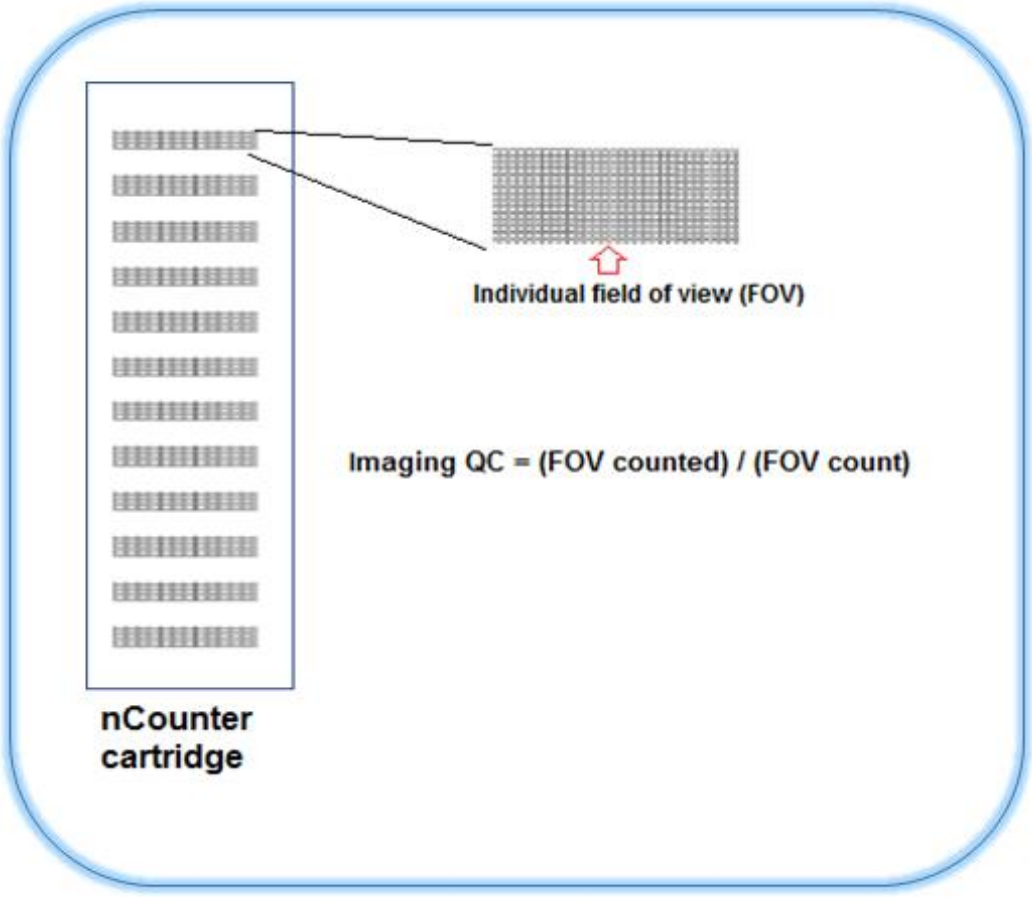


Figure 3.4 The image of a SPRINT cartridge showing the 12 gene counting areas and Imaging quality control calculation. Each counting area has several fields of view (FOVs).

(iii) Binding density QC

The binding density is the number of fluorescent spots per square micron that are bound to the slide surface. The imaging unit counts individual unambiguous molecules. Signal saturation (overlap of an image) can affect the linearity of the assay. The binding density is assessed after the experiment has been run. By assessing the binding density, future sample inputs can be adjusted for future runs. The amount of RNA loaded in the assay can be affected by the number of targets being quantified and expression level of certain highly expressed genes. Degraded RNA can affect the

value of binding density. The binding density of the samples should lie between 0.1-2.25 for the MAX/FLEX instrument and 0.1-1.8 for the SPRINT analyser.

(iv) Limit of detection

The limit of detection of the nCounter was checked for each assay. Samples passed this QC if the positive control count for (POS_E) was higher than the mean + 2SD of the eight negative control counts (Figure 3.5).

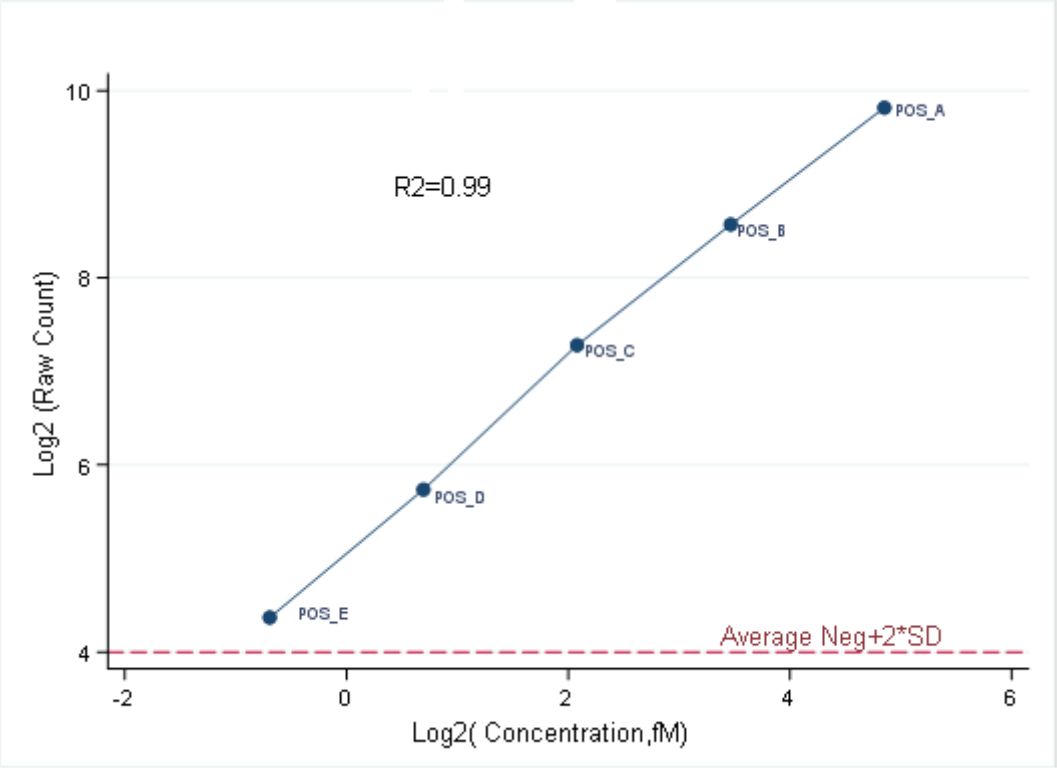


Figure 3.5 Linearity of positive controls and positive control limit of detection

(the lowest positive control POS_E is higher than the average negative (Neg) control counts plus 2 times standard deviation (SD) of the negative control count (red line).

3.2.16 RT-qPCR for *fhbP*

To evaluate the NanoString gene expression results for the *fhbP* gene, the same samples were analysed by RT-qPCR to cross-validate the transcript detection and count result obtained from the NanoString SPRINT.

3.2.16.1 cDNA synthesis using reverse transcriptase

cDNA of Nm was prepared by using the total RNA extracted using RNeasy mini kit and double digested on column with Qiagen DNA digest during extraction and off column using TURBO reagent. 48 samples were reverse transcribed to their cDNA and this template cDNA was used to assess the expression of different variants of fHbP.

A master mix was prepared by mixing 4µl DEPC (diethyl pyrocarbonate) water, 10µl 2x buffer (deoxyribonucleotide triphosphates (dNTPs), random oligonucleotides, buffer) and 1µl RT. Once the master mix was mixed properly, 5µl total RNA was added. The mixture was incubated in the thermocycler for one hour at 37ºC and 5 minutes at 98ºC and stored at -20ºC until needed.

3.2.16.2 qPCR reaction for fHbP variants

Two qPCR reactions were done using a protocol developed at the University of Leicester for the three different variants of fHbP. Variant 1 was done independently and Variants 2 and 3 were done together since the latter two variants have sequence homology that makes it difficult to design variant specific primers. A master mix was prepared by adding primers (forward and reverse) for the different qPCR reactions (variants), DEPC water, buffer and cDNA as explained in Table 3.1 and

Table 3.2 or Variant 1 and Variant 2 and 3 respectively. A control targeting a housekeeping gene (*gdh)* was run with the different qPCR reactions. The reaction was done in triplicate. A calibrator was included in every run.

Table 3.1 Master mix for Variant 1 fhbP qPCR

Reagent	**Concentration (µM)**	**Volume for one reaction (µl)**	**Final concentration (nM)**
Fast SYBR green	2x	5.00	1x
fhbP forward primer	10	0.40	400
fhbP reverse primer	10	0.40	400
fhbP probe	10	0.20	200
gdh forward primer	10	0.20	200
gdh reverse primer	10	0.20	200
ghd probe	10	1.45	200
water	NA	1.45	NA
Sample (cDNA)	NA	2.00	NA

Table 3.2 Master mix for Variants 2 and 3 qPCR

Reagent	**Concentration (**µM)	**Volume for one reaction (µl)**	**Final concentration (nM)**
Fast SYBR green	2x	5.00	1x
fhbP forward primer	10	0.30	300
fhbP reverse primer	10	0.30	300
fhbP probe	10	0.20	200
gdh forward primer	10	0.20	200
gdh reverse primer	10	0.20	200
ghd probe	10	1.45	150
water	NA	1.45	NA
Sample (cDNA)	NA	2	NA

Table 3.3 The real time PCR programme for Variant 1 and Variant 2 and 3 together

Step type	**Temperature**	**Time**	
UNG	50ºC	2 minutes	
Activation	95ºC	20 seconds	
Denaturation	95ºC	3 seconds	50cycles
Annealing /extension	58ºC	30 seconds	

Table 3.4 qPCR primers for Variant 1 and Variant 2 and 3 together information

Primer name	Target gene	Direction	Primer/probe sequence	Reference
FHbp_229C_F	*fhbP variant1*	*forward*	ACTTTATCCGCCAAATCGAAGT	Cayrou et al. 2018 (72)
FHbp_229T_F		*forward*	ACTTTATCCGTCAAATCGAAGT	Cayrou et al. 2018 (72)
FHbp_313_R		*reverse*	GCGGAATGGCTTTGTTTGTA	Cayrou et al. 2018 (72)
FHbp_253_P		*probe*	[6FAM]ACGGGIAGCTCATTACCTTGGAGA[BHQ1]	Cayrou et al. 2018 (72)
gdh350F	*gdh*	*forward*	TCGCCATTAAAGCCGAAATC	Cayrou et al. 2018 (72)
gdh416R2		*reverse*	TTGCCGGTACGCAGGTAGA	Cayrou et al. 2018 (72)
gdh374T		*probe*	[JOE]ACGAACGCTGGAAGGGCGTTC[BHQ1]	Cayrou et al. 2018 (72)
FHbp_system_2_586F	*fhbP variant 2 and 3*	*forward*	GCCGAACTCAAAGCAGATGA	Christopher D. Bayliss lab, University of Leicester (unpublished)
FHbp_system_2_636PR		*reverse*	[6FAM]CGTGTCGCCCAAAATGACGGC[BHQ1]	Christopher D. Bayliss lab, University of Leicester (unpublished)
FHbp_system_2_671R		*probe*	AGGTGGTAAGTGCCTTTTTCTT	Christopher D. Bayliss lab, University of Leicester (unpublished)

3.2.16.3 A standard for RT-qPCR

A standard was prepared from a synthetic sequence of nucleotides and copy number was determined by the concentration of the standard added in different dilutions (from 6 to 60,000 copies). A standard run was done for each run for both Variant 1 and Variants 2 and 3. The lower limit of detection is 100 copies/µl.

3.2.17 *sodC* qPCR to check DNA contamination of RNA samples

SodC qPCR was done to test for contaminating DNA in the total RNA pharyngeal samples extracted using RNeasy mini kit. On column DNA digestion was done for those samples.

A pre-prepared mix containing 300nM of Nm *sodC* forward and reverse primers, 100nM Nm *sodC* FAM and molecular grade water stored at -20ºC was thawed at room temperature. A master mix was prepared in a clean biosafety cabinet by adding 5µl of this pre-prepared mix to 10µl of tag polymerase. This master mix (15µl) was loaded on to a QIAgility pipetting robot and pipetted into each of the test wells on a 96 well microplate. 5µl of three positive and negative control samples were added to specific control wells, and 5µl total RNA samples thawed at room temperature were pipetted manually into all the other wells making a final reaction volume of 20µl in all the wells. The plate was sealed and spun for 3 minutes at 1800*g*. The qPCR was done using QIAsyphony real time PCR machine according to the qPCR programme shown in (Table 3.5).

Table 3.5 sodC qPCR programme

Step type	Temperature	Time	Number of cycles
UNG	50ºC	2 minutes	-
Activation	95ºC	20 seconds	-
Denaturation	95ºC	3 seconds	-
Annealing /extension	60ºC	1 minute	50

Table 3.6 sodC PCR primers and probe

Gene	Primers/ Probe name	Sequence (5'-3')	References
sodC	Nm-sodC-F351	GCACACTTAGGTGATTTACCTGCAT	Finn et al (65)
	Nm-sodC-R478	CCACCCGTGTGGATCATAATAGA	Finn et al (65)
Probe	Nm-sodC-P387 FAM	[6FAM]CATGATGGCACAGCAACAAA TCCTGTTT[BHQ1]	Finn et al (65)

3.3 Data Analysis

All analyses were done using Stata 15 software. Detail on specific analyses is given in the relevant chapters.

Chapter 4. Development of RNA extraction methodology and gene expression profiling of Nm from culture samples using NanoString nCounter technology

4.1 Introduction

Analysis of gene expression is becoming more widely used as a method to understand the lifecycles of bacteria and the pathogenesis of the diseases they cause. Transcriptomic analysis of *in vivo* pharyngeal samples of Nm to measure levels of gene expression relating to vaccine proteins could help to predict whether such vaccines might interfere with colonisation and lead to herd protection. It may also lead to the discovery of novel vaccine candidates with a potential impact upon acquisition and clearance of Nm.

The development of an effective gene expression profiling platform is necessary for detection of Nm transcripts from pharyngeal samples. Gene profiling from such samples is particularly challenging due to the presence of a mixed population of bacteria and the low density of Nm in a high percentage of pharyngeal carriers (65). One potential method to overcome these challenges is the use of direct gene transcript detection and quantification technology, the NanoString nCounter system, which has been shown to detect and quantify mRNA from low quantity samples accurately (103). NanoString, a digital direct nucleic acid counting technology, uses fluorescently colour coded barcodes and microscopic imaging to count up to 800 different transcripts in a single hybridisation reaction (3.2.15.2). Probe pairs are used to capture and detect

individual molecules, in this case mRNA transcripts, from a given sample. This method can directly detect and quantify gene transcripts from mixed bacterial samples using species-specific nucleotides without the need for bacterial isolation, cDNA synthesis or amplification (102, 103, 111, 112). This reduces error by avoiding the need to amplify the genes or enzymatic processing of samples, reducing sample handing time, and reducing the input of total RNA which is often low quantity in *in vivo* samples(113, 114). NanoString technology has been successfully used to profile other bacteria such as *Staphylococcus aureus* (102, 111, 114) but no published reports of its use to measure gene expression in Nm have been found.

Bacterial RNA purity and integrity alongside adequate yield is critical for successful gene expression assays and requires high quality RNA extraction. Most methods used for RNA extraction are designed for high density pure *in vitro* samples. For transcriptomic analysis, the extraction method is key to obtaining intact high-quality intact RNA that is free of RNase, protein and DNA. RNA has been successfully extracted from *in vivo* samples of pneumococci using acid:phenol:chloroform (115-119). An alternative method is the use of commercially-available RNA extraction kits such as RNeasy, which has successfully been used for Nm *in vitro* samples (95, 120)

Optimising RNA extraction from Nm cultures and evaluating the capacity of the NanoString system to measure mRNA from Nm isolates grown under different environmental stresses, when up and down regulation of different genes would be expected, are necessary developmental steps before attempting to measure gene expression in pharyngeal swab samples.

4.2 Aims

To optimise RNA extraction methods from Nm culture isolates and to evaluate the NanoString nCounter gene profiling technology to measure gene expression in cultured samples of Nm.

4.3 Objectives

- To select Nm genes for gene expression studies
- To produce a growth curve of Nm to identify the mid logarithmic phase for analysis
- To establish methodology for extraction and quantification of RNA from isolates of Nm cultured from meningococcal carriers
- To assess the feasibility of using different sample types (lysate and total RNA) for gene extraction
- To establish mRNA transcript detection assays (NanoString)
- To compare the gene expression profiles of meningococci cultured under different environmental conditions, thus confirming the potential to detect changes in expression.
- To evaluate performance of the system in detection and quantification of Nm gene transcripts from culture samples

4.4 Methods

4.4.1 Gene selection

A literature review was done to select genes that encode proteins of the two MenB vaccines, other surface expressed proteins that are potential vaccine candidates, and proteins with an important role in colonisation and metabolism of Nm. A search was done in December 2015 for published papers using MedLine and Google Scholar with search terms such as gene expression, gene profiling, transcriptomics, carriage density, Neisseria, meningococcus, meningococcal vaccines.

4.4.2 Probe design

The list of gene targets was sent to NanoString Technologies (Seattle, USA) for gene CodeSet design. A unique pair of capture and reporter probes of 100bp length (50bp attached to a biotin molecule and 50bp attached to a colour barcode respectively) complementary to the target sequence of the gene of interest was designed for each gene to make a CodeSet by the NanoString bioinformatics team. The capture probe attaches the complex of target sequence and probe mixture through a biotin molecule to a streptavidin coated cartridge, and the reporter probe through a colour barcode signals capturing of the transcripts. The probes for each mRNA target were designed to target as many isoforms as possible for all the genes.

The probes were screened against the human genome and all available neisseria transcriptomes (*N. gonorrhoeae, N. lactamica, N. elongata, N. wadsworthii, N. macacae, N. cinerea, N. bacilliformis and N. subflava*) for potential cross-reactivity. Screening was also undertaken for hybridization efficiency, potential for cross-hybridization, GC content and predicted secondary structure. All the sequences were

cross-checked by aligning the 100nt sequences in pubMLST and refseq databases with all the alleles present at the time. After the best scoring probes for the targets were selected by NanoString, a CodeSet Design Report was sent to me for review. The hybridisation efficacy of the probes was tested and certified by the company as having a high correlation (R^2 >0.95). NanoString Technologies then manufactured the CodeSet.

4.4.3 Growth curve

Colonies of Nm were suspended in RNase-free PBS-B, optical density (OD) measured at 600 nanometres (OD_{600} = 1). The bacterial suspension was inoculated into 10ml BHI broth supplemented with 10% horse blood and 2% isovitalex enrichment in 1:100 dilution. The experiment was run in duplicate. The liquid culture was incubated at 37°C with 5% CO_2 and shaken at 150rpm. Growth of Nm was monitored every two hours by measuring optical density of 1ml samples of the broth culture at OD_{600} using (Denovix D-11+ spectrophotometer). A growth curve was plotted by taking the mean of the duplicate measurements.

4.4.4 RNA extraction and optimisation

Two RNA extraction methodologies were compared for the yield and quality of total RNA from Nm cultures: the acid:phenol:chloroform method, which had successfully been used to extract pneumococcal RNA from *in vivo* samples, and a commercially available RNeasy mini kit.

Nm ATCC_BAA-335 strains were cultured on CBA agar and a suspension of the organism prepared as explained in section 3.2.2.2. The suspension was inoculated in broth culture, as for the growth curve, in four different tubes. 1ml samples were

collected at three different points during growth: early log phase (2 hours) and mid-log phase (4 and 6 hours) from each of the four tubes. The OD_{600} was measured at each point. The cultures were pelleted immediately to remove the culture media, suspended in 20µl RNase free PBS before adding 1ml RNAlater, a stabilising reagent, and stored at -80°C until RNA extraction.

Total RNA was extracted from the stored samples using (i) Qiagen RNeasy protect mini kit, and (ii) the method adopted from Ogunnyi et al using acid:phenol:chloroform (115, 117). All glassware was washed and decontaminated to avoid ribonuclease contamination (3.2.1). RNase free filter tips, test tubes and solutions were used in all experiments.

4.4.4.1 RNeasy mini kit

Bacterial RNA was extracted following the RNeasy mini kit procedure (3.2.5). On-column DNase digestion (3.2.6) was performed for all the samples.

4.4.4.2 Acid phenol chloroform

Samples collected into RNAlater were mixed with an equal volume of pre-warmed (65°C) acid:phenol:chloroform (125:24:1, ph 4.5) and were incubated in a heat block for 5 minutes at 65°C with vortexing at the mid-point and at the end of the incubation. The mixture was cooled on ice for one minute before centrifugation at 15,500*g* for one minute. Samples were phase separated and the aqueous phase was re-extracted with 500µl of warm acid:phenol:chloroform. Sodium acetate (40µl) at a concentration of 300µM and 2µl glycerol were added to the supernatant. Two volumes of absolute ethanol were added to precipitate the RNA overnight at -80°C. On the following morning, the samples were washed twice with 70% cold ethanol and DNase digestion

was performed using RQ1 RNase free DNase, 10x DNase buffer and RNasin® Ribonuclease Inhibitor. RNA clean-up was subsequently performed to improve RNA quality using RNeasy minElute clean-up kit.

For both methods, RNA quantity and quality were measured using the NanoDrop (Denovix D-11+) spectrophotometer. Absorbance of 1µl samples was measured at 260nm and 280nm. An optical density ratio A260/A280 of approximately 2 was taken to indicate good quality RNA (3.2.14.1).

4.4.5 Bacterial lysate and total RNA preparation

Since the sensitivity of detecting transcripts from a crude lysate could eliminate the need to go through the process of RNA extraction, gene expression from a crude lysate preparation of Nm cultured in optimum conditions was compared with the gene count from total RNA extracted using RNeasy minikit.

ATCC-BAA-335 Nm strains were cultured on CBA. Three different broth cultures containing 10ml heat inactivated BHI were each inoculated with a suspension of Nm from the overnight culture. For each of the three broths, 1ml Nm culture was collected into two different tubes after 1, 2 and 3 hours of incubation. All the collected samples were kept in RNAlater until processing.

The lysate was prepared by resuspending Nm pelleted in lysozyme plus TE buffer and treating the suspension using RLT buffer (3.2.9). One set of samples underwent total RNA extraction using the RNeasy minikit, the other set went directly to hybridisation (Figure 4.1). 5µl samples of the total RNA and the crude lysate were hybridised at the same time using one master mix, and gene counting was done using a SPRINT analyser.

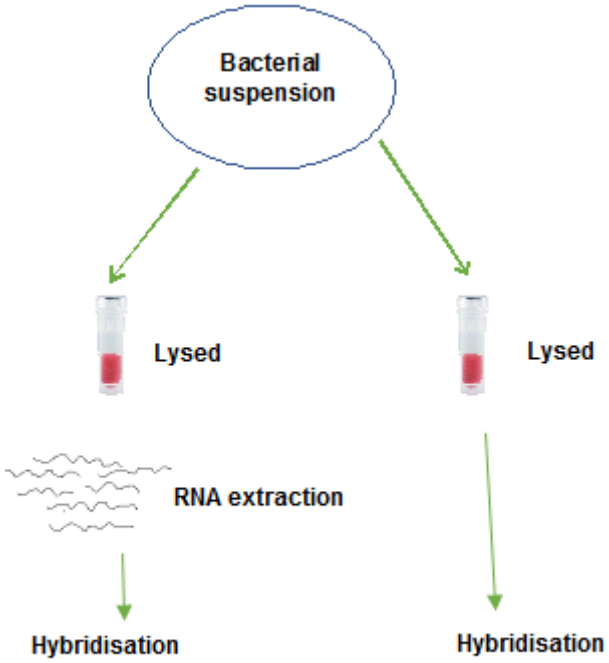


Figure 4.1 Workflow showing (i) RNA extraction from lysate and (ii) lysate alone before NanoString hybridisation

4.4.6 PrimeStore and RNAlater for Nm culture RNA extraction

Nm was cultured on CBA agar overnight and colonies were suspended in PBS-B to make a feeder culture (3.2.2.2). 100µl of the suspension was inoculated in 10ml BHI broth and left to grow to mid log phase (4 hours). Samples (1ml) were taken into eight different tubes. Four of the samples were stored in RNAlater and the other four were stored in PrimeStore. RNA was extracted using RNeasy mini kit and PrimeXtract.

4.4.7 Analysis of total RNA concentration from Nm broth culture samples collected in undiluted and diluted (1:2) RNAlater

A feeder culture was prepared, inoculated in BHI broth and left to grow to mid log phase (as 4.4.4.). Samples (1ml) were taken into four different tubes. Two samples

were store in undiluted RNAlater and the other two in a 1:2 diluted RNAlater. Total RNA was extracted using RNeasy mini kit.

4.4.8 Bacterial strain and culture conditions

Experiments were set up to assess performance of the nCounter system for Nm gene detection and technical reproducibility, and to measure changes in gene expression under environmental stress conditions (temperature variation) and nutritional stress (iron starvation) that Nm faces in the microenvironment of the host. The lower limit of detection, technical reproducibility of the platform, and fold change in expression were assessed for the genes selected from the review.

For temperature shock, standard ATCC-BAA-335 Nm strains were cultured on CBA at 37°C for 16 hours in 5% CO_2. Colonies were suspended in PBS-B to $OD_{600} = 1$ and inoculated at 1:100 dilution into 10ml BHI supplemented with 2% isovitalex and heat inactivated horse blood. At mid-logarithmic phase (OD_{600} = $0.5/8x10^8$), the liquid cultures were split into three and exposed to cold shock 26°C (to mimic the internal temperature of the nose when the environmental temperature falls to zero degree (121-123)), heat shock 40°C (to mimic the highest temperature that the human body can reach during infection (124) and 37°C as a control. The cultures were left to grow for another three hours at these temperatures. The broth cultures were collected and pelleted at 5,000*g* for 2 minutes and re-suspended in 20µl RNase free RNase free PBS. RNAlater (1ml) was added to the suspension and samples were left overnight at 4°C and stored at -80°C until RNA extraction. The experiment was done in triplicate.

For iron starvation, iron-defined GC media were used. Nm was grown on GC agar for 16 hours at 37°C in 5% CO_2. Colonies were suspended in PBS-B to $OD_{600} = 1$ and inoculated at 1:100 dilution into 10ml GC broth containing 12.5µM $Fe(NO_3)_3$ and 2%

isovitalex supplement. The experiment was run in triplicate. The bacterial suspension was split into two 4 ml volumes at mid-log OD_{600} = 0.3 (after 5 hours of incubation). One 4ml sample was exposed to 100μM deferoxamine, an iron chelator and the other 4ml sample was used as a control without a chelator. Both cultures were grown for an additional 15 minutes before collecting the samples into RNAlater. The bacterial cells were pelleted by centrifugation for 2 minutes at 5000*g* and re-suspended with 20μl RNase free water. 1μl RNAlater solution was added to the resulting suspension, stored overnight at 4°C and transferred to -80°C until RNA extraction.

4.4.9 RNA extraction for the heat shocked /cold shocked and iron depleted and replete samples

Prior to RNA extraction, the stored bacteria were thawed on ice and diluted with an equal volume of ice cold 1xPBS to the RNAlater and centrifuged at 5000*g* for two minutes. The pellets were treated with 15mg/ml lysozyme plus Tris-EDTA buffer to lyse the bacteria cells for 20 minutes at room temperature, shaking for 11 seconds every 2 minutes. Bacterial RNA was extracted using the RNeasy mini kit following the manufacturer's protocol (section 2.2.4).

4.4.10 DNA digestion of the culture samples

DNA digestion was done on-column to remove double stranded DNA during RNA extraction process to all the samples following RNeasy mini kit procedure (3.2.6).

4.4.11 Sample preparation and shipment to University College London

Forty-eight RNA samples were prepared for analysis. Of these, 24 were generated from Nm cultured at 37°C for measurement of technical reproducibility of the

NanoString platform. To assess this, one sample was taken and split into two replicates (replicate 1 and replicate 2). Each of the samples was diluted four-fold from 100ng to 0.097ng/µl total RNA (100ng, 25ng, 6.25ng, 0.4ng, 0.097ng). The other 24 samples were from Nm cultures in iron restricted/ replete conditions (12 samples) and from the heat/cold shocked Nm cultures (12 samples). All the RNA samples were diluted to 100ng total RNA concentration in 5µl. The samples were diluted to the desired concentration using RNase free water, frozen at -80°C and shipped on dry ice to University College London for the NanoString assays.

4.4.12 Gene expression profiling using nCounter platform

Gene expression profiling of the 48 Nm culture samples was done on the University College London NanoString nCounter platform, with four runs of 12 samples/run on the prep-station and digital analyser. The technical replicates were run independently in duplicate (12 reactions at a time), followed by the heat and cold shock samples (12 each). The samples were tested on consecutive days using one batch of probes and reagents.

The methods below are explained in Chapter 3 (see specific sections)

Sample Hybridisation (3.2.15.2)

Transcript count (3.2.15.4)

Internal QC (3.2.15.5)

Image QC (3.2.15.5)

For fold-change expression analysis, the ratio of the $\log_2$ of treatment over control was calculated using nSolver software version 3.0, and visual plots were done using Stata 15 (109). Fold change was defined as the mean gene count from three replicates

divided by the mean of the control replicates. All gene counts were normalised for the three housekeeping genes. If the quotients were less than one, the reciprocal was taken and a minus sign was added for ease of reading (125). One-way ANOVA was used to assess the difference in gene counts between heat/cold treated and iron depleted /replete samples and controls. The log10 transformed data were used to plot the correlation graphs between the technical replicates. Bonferroni correction was done to adjust for the 47 genes tested, and $P<0.001$ taken as the cut off value for the significance.

4.4.13 Negative control run using pharyngeal swab samples

Nm-negative samples that did not generate any *sodC* qPCR amplification up to 50 cycles were selected from BrisMenNHC samples to estimate the amount of RNA that could be obtained from the pharyngeal swab samples and the specificity of the probes in detecting Nm genes. Twelve Nm-negative samples were randomly selected from individuals who had negative samples on two or more visits. Total RNA from these samples was extracted using the protocol optimised for Nm culture samples (3.2.5). The total amounts of RNA were measured using Nanodrop (3.2.14.1).

4.5 Results

4.5.1 Gene selection

Forty-seven Nm genes were selected for probe design using the following criteria (i) genes coding for proteins in current vaccines, (ii) genes which are potential vaccine candidates, (iii) genes which are important in colonisation and adhesion of the bacteria to the pharyngeal mucosa (Table 4.1 ,Table 4.2 and Appendix B).

(i) Genes coding for proteins in current vaccines

One of the main criteria for gene selection for mRNA transcript detection assays using NanoString technology was those coding for meningococcal proteins in the two recently licensed MenB vaccines (Bexsero and Trumenba). The vaccine coverage of proteins expressed by high density carriers may be important in understanding the effectiveness of these vaccines in preventing transmission.

Three main components of Bexsero (28) were identified through reverse vaccinology as conserved proteins that generate bactericidal antibodies (i) Factor H binding protein (fHbp) a surface exposed lipoprotein which binds to human factor H, an inhibitor of the alternative complement pathway (ii) Neisseria adhesin A (NadA), a pathogenicity factor with a role in host cell adhesion and invasion (iii) Neisserial heparin-binding antigen, a lipoprotein which binds heparin and protects against complement-mediated killing. The fourth component consists of outer membrane vesicles whose dominant antigen is PorA. PorA is known to be highly variable, but important in generating bactericidal antibodies and a component of previous outer membrane vesicle vaccines (126). The Trumenba vaccine is made up of two fHbp protein variants from subfamilies A and B (24). Carriage of Nm serogroups B, C, W, Y is, or has been, frequent in the UK, and ACWY quadrivalent vaccine was introduced in 2015 for UK teenagers. The genes involved in capsule biosynthesis for serogroups B, C, W, Y have been recently reclassified (20).

(ii) Potential vaccine candidates

Other proteins have been proposed as vaccine candidates that are surface expressed and may generate bactericidal immunity. There is less certainty about the ability of these antigens to generate bactericidal antibodies in humans though they may do so

in mice, and they may be more variable. Granoff published a review of MenB vaccines in 2010 (24), referring to a number of additional potential vaccine targets such as Neisserial surface protein A, Transferrin binding proteins, Opacity proteins, and FetA. Tsolakos et al did proteomic analysis of surface expressed proteins that reacted with immune sera from mice (127). They identified new possible vaccine antigens including Macrophage infectivity potentiator, NMB 1946, NMB 1468, NMB 1612, NMB 0888, PilP and PilE. Other possible vaccine genes have been found through reverse vaccinology. Genome-derived neisserial antigens GNA33 and GNA992 are highly conserved, and elicit bactericidal antibodies in mice (128). Other vaccine candidates have been put forward, for example, T cell stimulating carrier protein A that may enhance the effectiveness of vaccines by acting as protective immunogen (127) and Adhesin Complex Protein that is upregulated under iron-depleted conditions.

(iii) Proteins important in colonisation: adhesion

The first step in the colonisation process is adhesion, attachment of bacteria to the epithelium of the nasopharynx, a coordinated process mediated through Type IV pili. Over 20 proteins are required including PilE, PilQ, PilV, PilN, PilO, PilC (93, 96, 129) (130). Other components play a role in bonding of Nm to the pharyngeal cells such as LPS and PorA. The opacity proteins Opa and Opc promote intimate adhesion. Opa binds carcino-embryonic antigen cell adhesion molecules (CEACAMs), heparin sulphate proteoglycan and integrins (36). Opa proteins are able to elicit innate human defences that favour the survival of Nm, while actively suppressing adaptive immune responses that would eliminate pathogens (131). Opc binds to host extracellular matrix proteins such as vitronectin as also does meningococcal surface fibril (Msf). They inhibit complement mediated killing and help meningococcal survival during colonisation (132). Other proteins thought to play an important role in establishing

carriage include Neisseria hia/hsf homologue A (NhhA), Adhesion and penetration protein (App), NadA, and Heat shock protein A (HspA) (129).

4.5.1.1 Proteins important in colonisation: adapting to the host

The ability to cope with the hostile microenvironment of the host by responding to limitations of oxygen and nutrients in the nasopharynx allows the bacteria to adapt to the ecological niche. For example, meningococci can grow in oxygen limited environments using nitrite as an alternative substrate. Temperature can act to trigger immune evasion by Nm as demonstrated by Loh et al who identified three thermosensor regions necessary for capsule biosynthesis, the expression of factor H binding protein and sialylation of lipopolysaccharide (133).

Jamet et al identified four genes that were needed for bacterial adaptation to host cells (*narP, estD, fadD1, nnrS*), mostly with a role in adapting to hypoxic stress during colonisation (Jamet 2013). Together with *narP, NMA 0797* and *NMA0798* are part of a two component system required for colonization of host cells (134-136).

Iron is a basic element needed for Nm to survive in the host, but no extracellular free iron is available in the body of the host, being bound to specific proteins. Growth of Nm in iron-regulated conditions has been shown to be associated with the up or down regulation of 235 genes with a high percentage of the genes upregulated. Virulence genes involved in iron uptake include f*bpB, tonB, lpbP, and expB* (93, 96). Other genes involved in iron acquisition (h*puA, hmbR*) show phase variation in persistent meningococcal carriers (137). Transcriptional regulation is controlled through the ferric uptake gene *fur* (94).

Alamro also showed phase variation during carriage in six other gene loci, namely *porA, opc, feta, nadA, mspA,* and *nalP*. The last two are autotransporter genes. Early changes in transcriptomes on adhesion to epithelial cells are seen in genes that induce sulphate ABC transporter genes *cysW, cysA* and *cysT* (138). The regulator gene *NMB0573* controls the response to nutrient availability and has an important role in colonisation as an indicator of general amino acid abundance (138). Another gene that is critical for adaptation to the host is *xseB* (139) responsible for an inducible repair system that upregulates when Nm interacts with host cells.

4.5.2 Probe design

Probes were designed for the 47 selected genes and three housekeeping genes. Of the 47, 31 did not have any cross reactivity with other neisseria species or other organisms, but it was not possible to design a species-specific probe for 16 genes. The CodeSet design report is shown in Table 4.1 and Table 4.2.

Table 4.1 Neisseria meningitidis genes and target sequences of NanoString probes (n=50 including three housekeeping genes), Tm CP (temperature of capture probe), Tm RP (temperature of reporter probe)

Ser. NO	Customer Identifier	Accession	Position	Target Sequence	Tm CP	Tm RP
1	*fhbP*	fhbP_allele_100.1	89-188	GTTTGCAGTCTTTGACGCTGGATCAGTCCGTCAGGAAAAACGAGAAACTGAAGCTGGCGGCACAAGGTGCGGAAAAAACTTATGGAAACGGCGACAGCCT	81	85
2	*nadA*	NMB0394.1	606-705	CGAAACGGGCGCGGATATGCTGTTGTGGCAGGGTTCGTGCATCGTTCACAACGAATTCAAAGGGCAAGAGCTGGCGGCGTTGAAGGCGGAACACCCCGAA	84	84
3	*nhba*	NMB2132.1	598-697	AGCAATTTTGGAAGGGTTGATTTGGCTAATGGCGTTTTGATTGACGGGCCGTCGCAAAATATAACGTTGACCCACTGTAAAGGCGATTCTTGTAGTGGCA	78	79
4	*porA*	NMB1429.1	392-491	CGAATCAGTTTGACGATGCCAGCCAAGCCATTGATCCTTGGGACAGCAATAATGATGTGGCTTCGCAATTGGGTATTTTCAAACGCCACGACGACATGCC	83	80
5	*cssA (synX)*	NMB0070.1	308-407	AAGCACTAGCAGGCGCAGCTGTAGGTGCATTAAGCAGCCGTTTAGTTTGCCATATCGAAGGTGGTGAACTATCTGGTACAGTAGATGACTCCATTCGTCA	84	80
6	*cssB (siaB)*	NMB0069.1	265-364	GCTTTAGAAACAATTGGCAGTAATTCCGGCACAGTAACCCTATTACAACCAACCAGTCCATTACGCACAGGGGCTCATATTCGTGAAGCTTTTTCTCTAT	79	77
7	*cssC (siaC)*	NMB0068.1	584-683	TGGGTGGTATGAACGATTTATCTGAAGCCTTTCCAGACGCAATCATTGGCCTGTCTGACCATACCTTAGATAACTATGCTTGCTTAGGAGCAGTAGCTTT	80	79
8	*GNA1030*	NMB1030.1	428-527	TGGAGAAAACCGAAGTTTGTGGCGGCGACTTCAGCACCACCATCGACCGCACCAAATGGGGCATGGACTACCTCGTTAACGTTGGTATGACCAAAAGCGT	85	85
9	*nspA*	NMB0663.1	304-403	GGCGCGCGCTTGAGCCTCAACCGCGCCTCCGTCGACTTGGGCGGCAGCGACAGCTTCAGCCAAACCTCCATCGGCCTCGGCGTATTGACGGGCGTAAGCT	86	85
10	*tbpA*	NMB0461.1	162-261	AGTAACCGGGCTGGGCAAGTTGGTCAAGTCTTCCGATACGCTAAGTAAAGAACAGGTTTTGAATATCCGAGACCTGACCCGTTATGATCCGGGTATTGCC	84	80

11	*tbpB*	NMB0460.1	1538-1637	TGAAATACGGAATGTTGACGCGCAAAAACAGCAAGTCCGCGATGCAGGCAGGAGAAAGCAGTAGTCAAGC TGATGCTAAAACGGAACAAGTTGAACAAAG	86	85
12	*fetA*	NMB1988.1	1298-1397	TGCGTTACGACCGCTTCAAGGTGAAAACCCACGACGGCAAAACCGTTTCAAGCAACAACCTTAACCCGAGT TTCGGCGTGATTTGGCAGCCGCACGAACA	83	84
13	*opc*	NMB1053.1	406-505	GAATCTTCAAAAGACAGCATTAAAACCACCAAGCATACGCTTCACAGCAGCCGTCAGTCGTGGTTAGCCAA AGTTCACGCGGATTTGCTTTCCCAACTGG	82	79
14	*mip*	NMB1567.1	248-347	AAGCCATGCAGGCAGTGTATGACGGCAAAGAAATCAAAATGACCGAAGAGCAGGCTCAGGAAGTCATGAT GAAATTCCTTCAGGAACAACAGGCTAAAGC	85	86
15	*NMB1946*	NMB1946.1	402-501	ATCGCTGGAAGAAGTCAAAGACGGCAGCACCGTATCCGCGCCCAACGACCCGTCCAACTTCGCCCGCGTC TTGGTGATGCTCGACGAACTGGGTTGGATC	87	84
16	*NMB1612*	NMB1612.1	370-469	ACCCAAGTCGTCCTCGTTCCGAAAGGCAAAAAAGTATCTTCTTCCGAAGATTTGAAAAACATGAACAAAGTC GGCGTGGTAACCGGCTACACGGGCGATT	79	84
17	*NMB0088*	NMB0088.1	558-657	ATATGCCGACTGGGGGATTAAGAGTAAAGCAGAGATATTGACGGCAAAACCGCCCAAACCTAACGGTGTAG CCGAAGCTGCAAAAATTCAGGCCGACGGA	86	86
18	*pilP*	NMB1811.1	356-455	CTGTCGGTGTCGGCAACTATTTGGGACAAAACTACGGTAGAATCGAAAGCATTACCGACGACAGCATCGTC CTGAACGAGCTAATAGAAGACAGCACGGG	83	85
19	*pilE*	NMB0018.1	41-140	TGATTGTGATTGCCATCGTCGGCATTTTGGCGGCAGTCGCCCTTCCTGCTTATCAAGACTACACAGCCCGC GCACAAGTTTCCGAAGCCATTCTTTTGGC	81	81
20	*dsbA-2*	NMB0294.1	352-451	GCCGCAGTGGAAATGGCCGGTGAATCAGATAAAGCCAACAGCCATATTTTCGATGCGATGGTTAATCAAAA AATCAATCTGGCCGATACCGATACCCTGA	84	80
21	*GNA33*	NMB0033.1	842-941	ACAAAAACGAACATCCCTACGTTTCCATCGGACGCTATATGGCGGATAAGGGCTACCTCAAACTCGGACAA ACCTCCATGCAGGGCATTAAGTCTTATAT	81	84
22	*tspA*	NMB0341.1	1819-1918	GAAGAGTTGCACGATTTCCTGAAAGTGTACGAAACCGATGCCGTCGCGGAAACTGCGCCTGAAACGCCCG ATTTCAACGCCGCCGCAGACGATTTGTCCG	83	85

23	*msf*	NMB0992.1	548-647	GTTCGACTTTGACCGATACGCTGCTGAATACCGGAGCGACCACAAACGTAACCAACGACAACGTTACCGAT GACGAGAAAAAACGTGCGGCAAGCGTTAA	84	84
24	*pilC1*	NMB1847.1	1120-1219	CCAAATTTTCCTGCAAAACCTTTCCCGCAAGGATGACACAAGCAAACCGGGCCGCTATTCCCTCAAACCCTT GAGTACGTCGGAGATTAAAAGTAAAGAG	82	81
25	*xseB*	NMB0262.1	88-187	GAAGACGCGCTTGCCGCCTATCAGGAAGGCAACGAACTGGTCAGGTACTGCCAAACCAAACTGGCACAAG TCGAACAAAAGCTACAGGTTTTAGACACAG	85	84
26	*nnrS*	NMB0439.1	448-547	TTCCACGTCCAGCTGCACAACGGCAACCTAGGCGGACTCTTGAGCGGATTGCAGTCGGGCTTGGTGATGG TGTCGGGTTTTATCGGTCTGATTGGTACGC	84	81
27	*estD*	NMB1305.1	396-495	CTTTCCTACCAACGGCAAACGTTCCATTATGGGACATTCAATGGGCGGACACGGCGCATTGGTATTGGCGC TGCGGAATCAGGAACGTTATCAAAGTGTT	82	81
28	*fadD1*	NMB1276.1	333-432	CGGCCTGTCAAAAGAATTGGCGGGCTTGAAGGCGCAAACGCCCGTCGAAAAAATCATTTGGACGGACAAA AGCCGTCCGACCGGCGAAACGGCGGAAGGC	85	85
29	*NMB1845*	NMB1845.1	109-208	GGTGCGGCAGGGCGGCCTTTGACCCTGCTGTCGGGGCAGCGGCTGACTTTGGGACAGTTTAGCCGAGAT AAGGCGGTTTTGGTGTATTTTTGGGGGAGCT	85	80
30	*cysW*	NMB0880.1	422-521	TCTTCGCCATCCCCGGTATTGTTTTGGCGACGCTGTTCGTTACCTTCCCCTTTGTCGCACGCGAAATCATCC CGCTGATGCAGGCACAGGGCGACAGCGA	77	85
31	*cysT*	NMB0881.1	481-580	CCGGTATTGGAAGAATTGTCGGGCGAATATGAGGAAGCGGCGGCAACTTTGGGCGCAAGCCGTTGGACTA CGTTTCGCCGTGTCCTCTTGCCTGAAATCA	84	84
32	*cysA*	NMB0879.1	867-966	ACCGATGATTTGTGCCGAAATCGAAAAAATCCACGCCGTCGGCGCATTGACGCATATTCTGGTAAAACACG ACAAACAGGACGTACATATCACGCTGGCA	81	83
33	*NMB0573*	NMB0573.1	397-496	ACCGACTACCTGCTTCAGGCGTTTTTTACCGATATGAACGCGTTTTCCCATTTTGTTTTGGATACGCTCCTGT CCCACCACGGCGTACAAGATGCGCAAT	76	80
34	*pilN*	NMB1809.1	307-406	AAAATCCTCGACAGCCTGAATGAGGCCGTCCCCGGAAGCACCTACCTGACCTCGCTGGATGCCGTTACCG CCGACTCTTATCGGCTCAGCGGCAGGACAT	89	85

35	*pilO*	NMB1810.1	324-423	TCATCAGGCAGGTTCGAGCAACGGTCTGCGCTTGGACAGCGTTATGCCCCAACCTCCCGTAGATGACGGC CCCATCAAAAGATTACCCTATTCCATTTCC	85	81
36	*pilV*	NMB0887.1	233-332	GCAACAAGAAAAACTATAATCTTTACATGGGAAACCATACACTATCAGCTGTGGATGGCGATTTTGCGATTG ATGCCATGAAAACTAAGGGGCAATTGGC	80	83
37	*app*	NMB1428.1	972-1071	CGAGTTTGAAGACATCATCGGCAACGGCGGCAGCCTGACCGAAATCGACGTGGACACCATGCTTTATCGC CACCGCAGCGTGCGCCCAGGCTTCATTTCA	85	84
38	*iga*	NMB0700.1	2224-2323	ATCACCGCCAACATCACCGCAACAGACAACGCCAAAGTAAATTTGGGTTACAAAAACGGCGATGAGGTTTG CGTGCGCTCGGACTATACCGGTTACGTTA	83	81
39	*fbpB*	NMB0633.1	1312-1411	GCCGCATTCGCACTCGTCTTCCTCAAACTGATGAAAGAGCTGACCGCCACCCTGCTGCTGACCACCGACGA TGTCCACACACTCTCCACCGCCGTTTGGG	85	87
40	*tonB*	NMB1730.1	282-381	GGATATTCAGCAGCCTAAGGAAGAGCCGAAACCTGAAGAAAAGCCGAAACCCGAAGAAAAACCGAAACCA GAGCCTAAGCCGGAAGCGAAGCCTGTCCCG	86	85
41	*lbpB*	NMB1541.1	560-659	GTCCTTCCCAATCTTTACCGAGCGCGGGAACGGTGCAATATTCCGGTAACTGGCAATATATGACCGATGCC AAACGTCATCGGACAGGTAAGGCGGTTTC	82	84
42	*csb*	NMB0067.1	369-468	TCATCTCTATATGCTGTCTTTTACAGGCCACTACTCCTATCTGATTAGTATTGCAAAAAAAAAGAATATTACGA CTCATTTAATTGATGAAGGGACTGGA	75	75
43	*frpC*	NMB1415.1	1015-1114	TTGGCTCAAGCCGCCAAAGAAGCATACGAAAATGCCAAATCCACAGCCGAGAAGGCTGCTCAAGCAGCTC GAGAATTTTTTAAGGGCTTGCCCAGTTTTA	86	78
44	*fur*	NMB0205.1	120-219	TGTGTACCGCATTTTGTTGGAAGAGGGTGTGGAAATCGGTGTGGCGACGATTTACCGTGTGCTGACCCAGT TTGAGCAGGCGGGCATTTTGCAACGCCAT	83	83
45	*aroE*	NMB0358.1	220-319	CTGGCGGACGAGCATTCCGAACGCGCATTGGCGGCAGGTGCGGTCAATACGCTGATTCCGTTGAAAAACG GCAAGCTGCGTGGCGACAACACCGACGGTA	84	85
46	*adk*	NMB0823.1	247-346	TTTGACGGTTTCCCGCGCACATTGGCACAAGCCGAAGCGATGGTTGAAGCAGGCGTGGATTTGGATGCAG TCGTTGAAATCGATGTGCCTGACAGCGTGA	84	81

47	*abcZ*	NMB1051.1	1167-1266	GTTCCGCAGCGCGTTGAATGAAAACGACACCGTGTTTTACACCCTCGGACAGGGAAACGATTACGTTGAAG TCGGCGGTAAGAAAAAACACGTCATGAGC	82	83
48	*hmbR*	NMB1668.1	768-867	TTTGGGTAAGATTGCTTACCAAATTAACGATAACCACCGCATCGGCGCATCGCTTAACGGCCAGCAGGGAC ATAATTACACGGTTGAAGAGTCTTATAAC	79	82
49	*opaD*	NMB1465.1	177-276	TGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTAAACACAAAAGAGTTGCAAAAAAACAATAGCAG TGGCATCTGGCAAGAACTGAAGACGGAA	78	85
50	*znuD*	NMB0964.1	501-600	GCCTGAAAACGGCGTATCGGGCGAACTCGGATTGCGTTTGAGCAGCGGCAATCTGGAAAAACTCACGTCC GGCGGCATCAATATCGGTTTGGGCAAAAAC	81	79

Table 4.2 NanoString probes for Neisseria meningitidis gene detection (n=50 genes including three housekeeping genes), Flags (Cross hybridization potential), HUGO gene (Common name for mRNA target sequence, as defined by RefSeq)

Ser.NO	Customer Identifier	**Accession**	Flags	HUGO Gene	Design Remarks
1	*fHbp*	fHbp_allele_100.1		*fHbp*	
2	*nadA*	NMB0394.1	X	*nadA*	will give signal for *N. gonorrhoeae* hits @95% - may give signal for *N. lactamica; N. elongata; N. bacilliformis* hits @93-94%
3	*nhba*	NMB2132.1		*NMB2132*	
4	*porA*	NMB1429.1		*porA*	
5	*cssA (synX)*	NMB0070.1		*synX*	
6	*cssB (siaB)*	NMB0069.1		*siaB*	
7	*cssC (siaC)*	NMB0068.1		*siaC*	
8	*GNA1030*	NMB1030.1	X	*NMB1030*	will give signal for *N. gonorrhoeae* & *N. lactamica* - hits @95%
9	*nspA*	NMB0663.1	X	*nspA*	will give signal for *N. lactamica* hits @95% and may give signal for *N. gonorrhoeae* hits @91%
10	*tbpA*	NMB0461.1		*tbp1*	
11	*tbpB*	NMB0460.1		*tbp2*	
12	*fetA*	NMB1988.1		*frpB*	
13	*opc*	NMB1053.1		*opc*	

14	*mip*	NMB1567.1	X	*NMB1567*	will give signal for *N. gonorrhoeae; N. lactamica; N. cinerea* - hits @95-97%
15	*NMB1946*	NMB1946.1	X	*NMB1946*	will give signal for *N. gonorrhoeae* & *N. lactamica* - hits @95%
16	*NMB1612*	NMB1612.1	X	*NMB1612*	will give signal for *N. gonorrhoeae* & *N. lactamica* - hits @97-98%
17	*NMB0088*	NMB0088.1		*NMB0088*	
18	*pilP*	NMB1811.1	X	*pilP*	will give signal for *N. gonorrhoeae* - hits @98%
19	*pilE*	NMB0018.1		*pilE*	
20	*dsbA-2*	NMB0294.1		*dsbA-2*	
21	*GNA33*	NMB0033.1		*NMB0033*	
22	*tspA*	NMB0341.1		*NMB0341*	
23	*msf*	NMB0992.1		*hsf*	
24	*pilC1*	NMB1847.1		*pilC1*	
25	*xseB*	NMB0262.1		*xseB*	
26	*nnrS*	NMB0439.1		*NMB0439*	
27	*estD*	NMB1305.1	X	*NMB1305*	may give signal for *N. gonorrhoeae; N. lactamica; N. elongata* - hits @93-94%
28	*fadD1*	NMB1276.1	X	*fadD-1*	also targets *N. gonorrhoeae* hits @97%
29	*NMB1845*	NMB1845.1	X	*NMB1845*	small risk of signal for *N. gonorrhoeae* & *N. lactamica* - GP will bind RP hits @88%

30	*cysW*	NMB0880.1	X	*cysW*	may give signal for *N. gonorrhoeae; N. lactamica; N. elongata* - hits @89-92%
31	*cysT*	NMB0881.1		*cysT*	
32	*cysA*	NMB0879.1		*cysA*	
33	*NMB0573*	NMB0573.1	X	*NMB0573*	will give signal for *N. gonorrhoeae* & *N. lactamica* - hits @95%
34	*pilN*	NMB1809.1		*pilN*	
35	*pilO*	NMB1810.1	X	*pilO*	will give signal for *N. gonorrhoeae* - hits @97%
36	*pilV*	NMB0887.1		*NMB0887*	
37	*app*	NMB1428.1		*NMB1428*	
38	*iga*	NMB0700.1		*iga*	
39	*fbpB*	NMB0633.1	X	*fbpB*	will give signal for *N. gonorrhoeae* & *N. lactamica* - hits @95%
40	*tonB*	NMB1730.1		*tonB*	
41	*lbpB*	NMB1541.1		*lbpB*	
42	*csb*	NMB0067.1		*NMB0067*	
43	*frpC*	NMB1415.1		*frpC*	
44	*fur*	NMB0205.1	X	*fur*	may give signal for *N. gonorrhoeae* & *N. lactamica* - hits @92%
45	*aroE*	NMB0358.1	HK	*aroE*	

46	*adk*	NMB0823.1	HK	*adk*	
47	*abcZ*	NMB1051.1	HK;X	*NMB1051*	small risk of signal for *N. gonorrhoeae* - GP will bind RP hits @88%
48	*hmbR*	NMB1668.1		*hmbR*	
49	*opaD*	NMB1465.1		*NMB1465*	
50	*znuD*	NMB0964.1		*NMB0964*	

**Hits percentage = the percentage closely related sequences

4.5.3 RNA extraction and optimisation

To determine the mid-logarithmic phase to inform sampling in subsequent experiments, a standard growth curve of Nm was obtained. The duplicate curves Figure 4.2 were very similar and the mid-logarithmic phase was taken as 5hours.

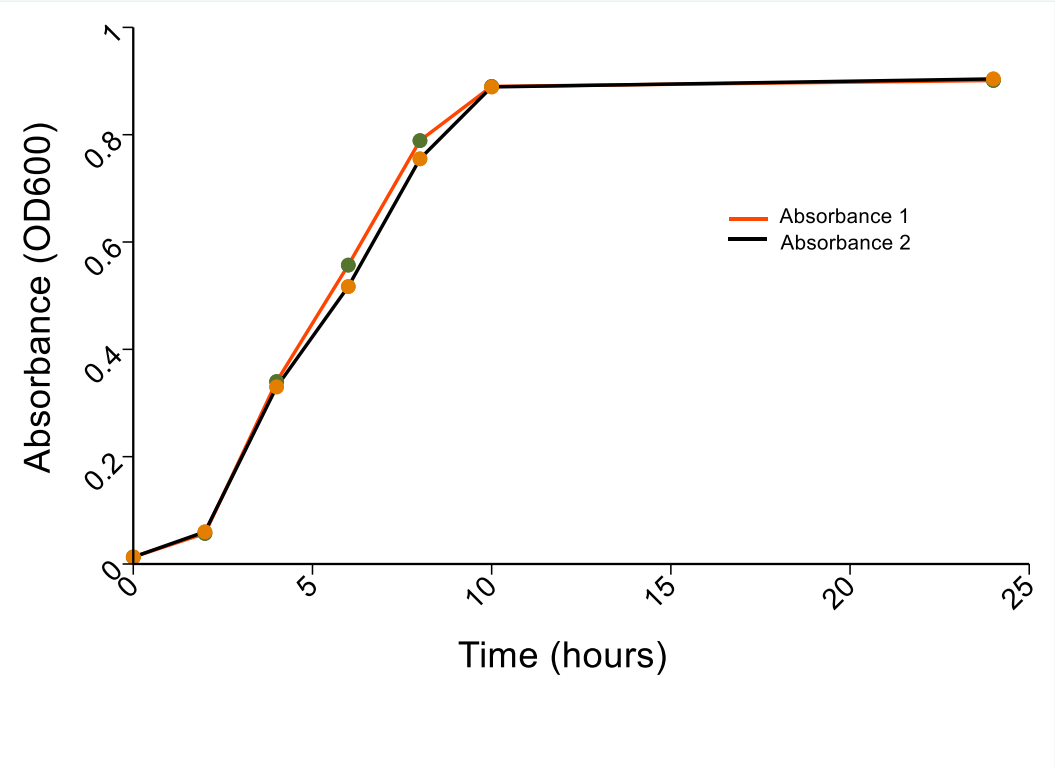


Figure 4.2 Duplicate growth curves of Neisseria meningitidis serogroup B strain ATCC_BAA_335

in brain heart infusion broth containing 10% heat-inactivated horse blood

4.5.4 Comparison of the quality and quantity of RNA purified using two methods

To decide the best method of RNA extraction for subsequent experiments, two established methods were compared. At OD_{600} 0.222 (2 hours) the acid:phenol:chloroform method yielded a mean of 5478.2ng/µl total RNA concentration compared with 67.1ng/µl using RNeasy mini kit from samples in RNAlater (Table 4.3) At OD_{600} 0.345 (4 hours) the mean yields were 3617ng/µl from the first method compared with 39.5ng/µl using the RNeasy mini kit. The acid:phenol:chloroform method gave lower purity RNA with absorbance ratios (A260/A280) of 1.6 at 2 hours and 1.6 at 4 hours, whereas the samples extracted using RNeasy mini kit had an absorbance ratio of approximately 2 for all samples, indicating higher purity RNA. The quantities of RNA obtained using the RNeasy mini kit were much less variable than those obtained using the acid:phenol:chloroform technique (Table 4.4)

Table 4.3 Quantity and quality of RNA extracted using acid:phenol;chloroform and RNeasy mini kit

Sample taken at	**RNA extraction methods**	
	Acid:phenol:chloroform	RNeasy mini kit (RNAlater)
2 hours growth	5478.2ng/µl (1.6)	67.1ng/µl (2.2)
4 hours growth	3617.0ng/µl (1.6)	*39.5ng/µl (2.2)

Each RNA value is an average of quadruplicate measures unless specified. The numbers in brackets are the nucleic acid absorbance ratio A260/A280. *n=2

Table 4.4 Variability of RNA concentration at 2 hours and 4 hours using acid:phenol;chloroform and RNeasy mini kit

	RNA extraction methods	
	Acid:phenol:chloroform	RNeasy mini kit (RNAlater)
RNA concentration (ng/µl) at 2hours	1657.4	93.9
	8130.5	72.5
	4735.2	54.7
	7389.7	47.2
Mean (standard error)	5478.2 (1467.3)	67.1 (10.3)
RNA concentration (ng/µl) at 4 hours	486.7	57.7
	272.9	21.3
	4918.9	ND
	8789.7	ND
Mean (standard error)	3617.0 (2029.8)	*39.5 (18)

ND-not done, *n=2

In view of the low quality and high variability of RNA obtained using the acid:phenol:chloroform method, RNA clean-up was subsequently done on the remaining samples for the same experiment (Table 4.5) **Error! Reference source not found.**The mean RNA concentration dropped from 501.5 to 8.5ng/µl, well below that in the higher purity samples obtained from the RNeasy mini kit but still with absorbance ratios indicating low purity.

Table 4.5 RNA quantity and quality using acid:phenol:chloroform, before and after clean-up using RNeasy minElute cleanup Kit

sample	RNA concentration(ng/µl) using acid:phenol:chloroform	
	Before clean-up	After clean-up
6 hours	426.9 (1.5)	7.5 (3.2)
	425.3 (1.5)	2.3 (25.2)
	842.7 (1.6)	17.1 (2.6)
	310.9 (1.4)	7.2 (3.8)

The numbers in brackets are the nucleic acid absorbance ratio A260/A280.

4.5.5 Comparison of bacterial lysate vs total RNA

To explore whether crude lysate could be used to measure gene expression instead of total RNA extracted using the RNeasy minikit, Nm genes were detected from both total RNA and the crude bacterial lysate using the nCounter SPRINT analyser. The gene counts from the total RNA extract were higher for all genes tested than from the equivalent crude lysate. The results were consistent for different concentrations of RNA or lysate tested (**Error! Reference source not found.**)

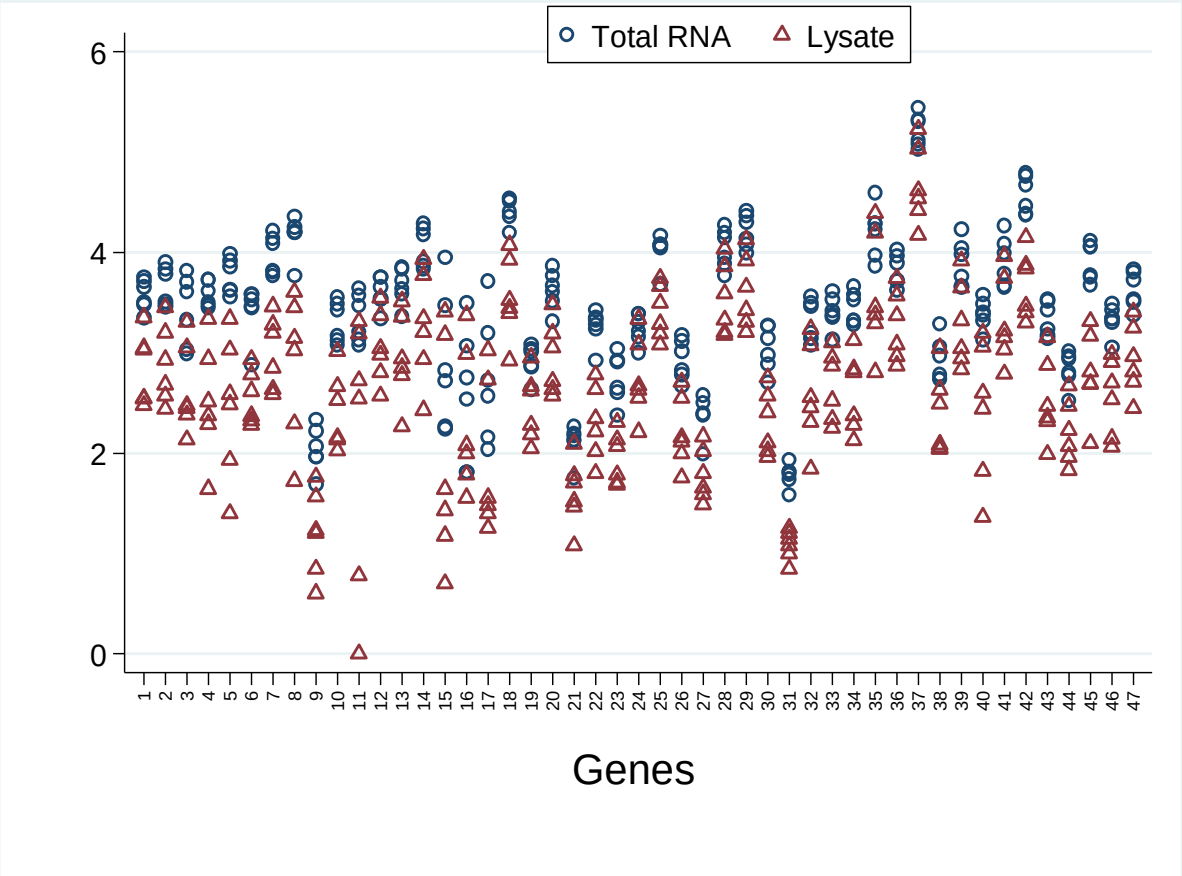


Figure 4.3 Comparison of gene counts from total RNA and crude lysate prepared from the same Nm culture samples.

The blue dots are counts from the total RNA extract and the red triangles are counts from the crude lysate of the same sample at different concentration of RNA

4.5.6 Comparison of diluted and undiluted RNAlater

To determine if dilution of RNA improved the yield of RNA using RNeasy minikit, RNA extraction was comparted using undiluted and diluted RNA. Undiluted RNAlater stored samples yielded approximately twice as much total RNA as 1:2 diluted RNAlater stored samples (Figure 4.4)

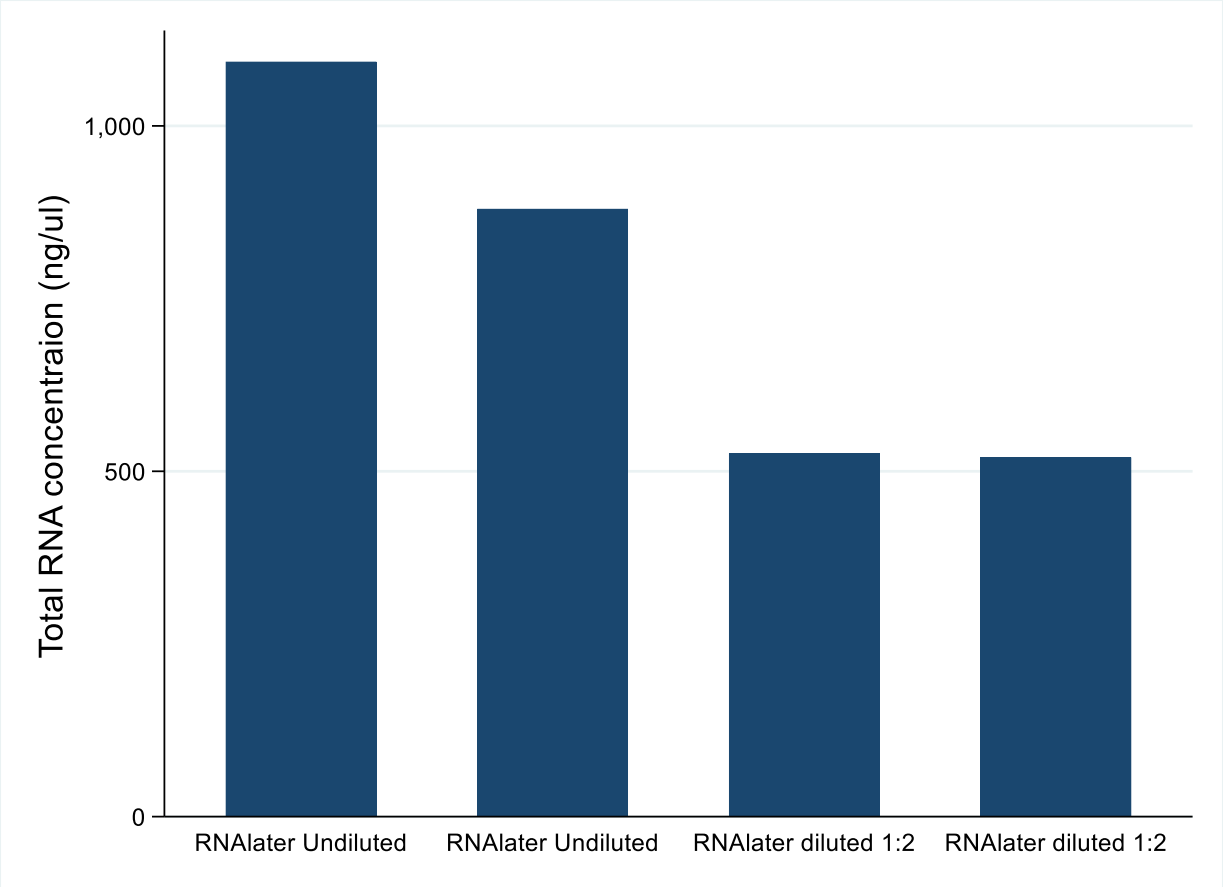


Figure 4.4 Analysis of total RNA concentration from Neisseria meningitidis broth culture samples collected in undiluted and diluted (1:2) RNAlater (n=1 for each bar)

4.5.7 Comparison of total RNA collected into two RNA storage media (PrimeStore and RNAlater)

RNA extraction was compared after storage in RNAlater and PrimeStore to determine the better storage media. RNA stored in RNAlater and extracted using RNeasy mini kit yielded a far higher quantity of RNA than from RNA stored in PrimeStore (Figure 4.5)**Error! Reference source not found.**. PrimeXtract yielded low quantities of RNA from both preservative media.

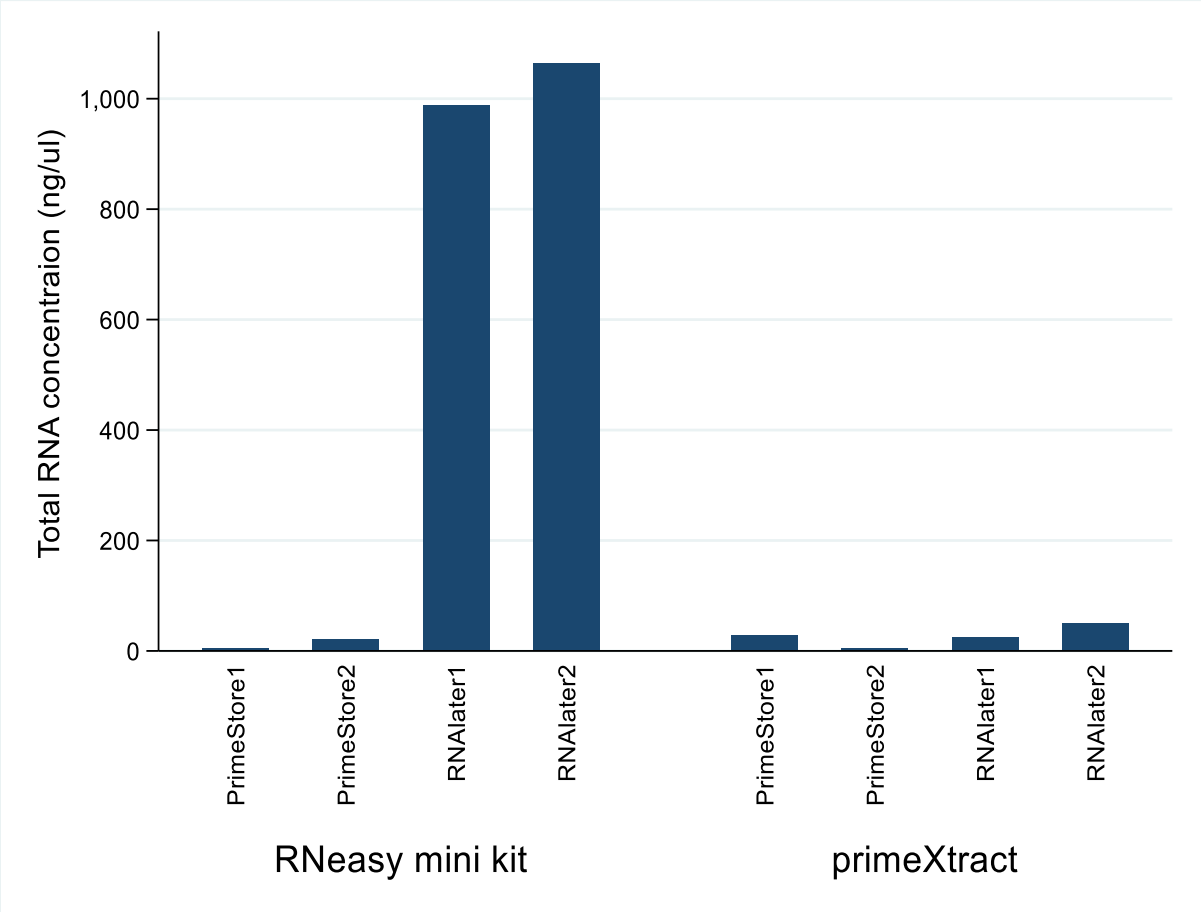


Figure 4.5 Analysis of total RNA collected into two RNA storage medias (PrimeStore and RNAlater) and extracted using two RNA extraction kits (PrimeXtract and RNeasy mini kit)(n=1 for each bar)

4.5.8 RNA extraction assessment from heat shock / cold shock culture samples

The quality and integrity of RNA extracted from the culture samples were confirmed by Nanodrop and a bioanalyzer (Agilent 2200 RNA tape station) (3.2.14.2). All the samples measured with RNA tape station had good quality and quantity with a value of >7.7 RIN (Figure 4.6).

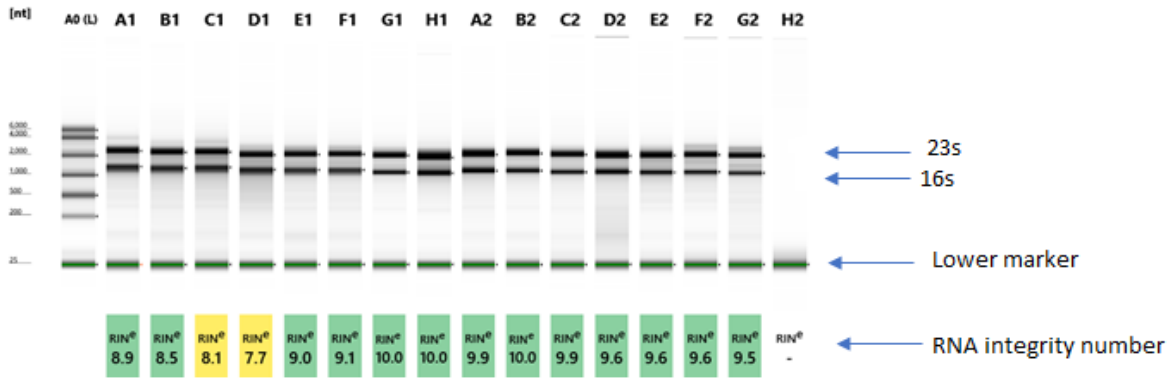


Figure 4.6 Gel image showing RIN number (RNA integrity) of Nm culture samples A1-C1 (iron replete), D1-F1 (iron depleted) G1-A2 (cold shock 26ºC), B2-D2 (control 37ºC), E2-G2 (heat shock 40ºC), using Agilent 2200 tape station system. The RIN score is highlighted in yellow and green. The three bands indicate the 23s and 16s RNA fragments and the lower marker.

4.5.9 Detection limit and technical reproducibility

The technical reproducibility and lower limit of gene detection of the nCounter platform was checked using Nm culture samples. A high degree of correlation between gene transcript counts of Nm from two technical replicates of 100ng RNA (Figure 4.7, R^2=0.99) was shown. Serial dilution of RNA preparations demonstrated that gene expression signals could be detected from RNA concentrations as low as 0.1ng/µl. The heat map below shows the similarity of the 47 gene counts from two technical replicates (Replicates 1 and 2) in logarithmic form (Figure 4.8).

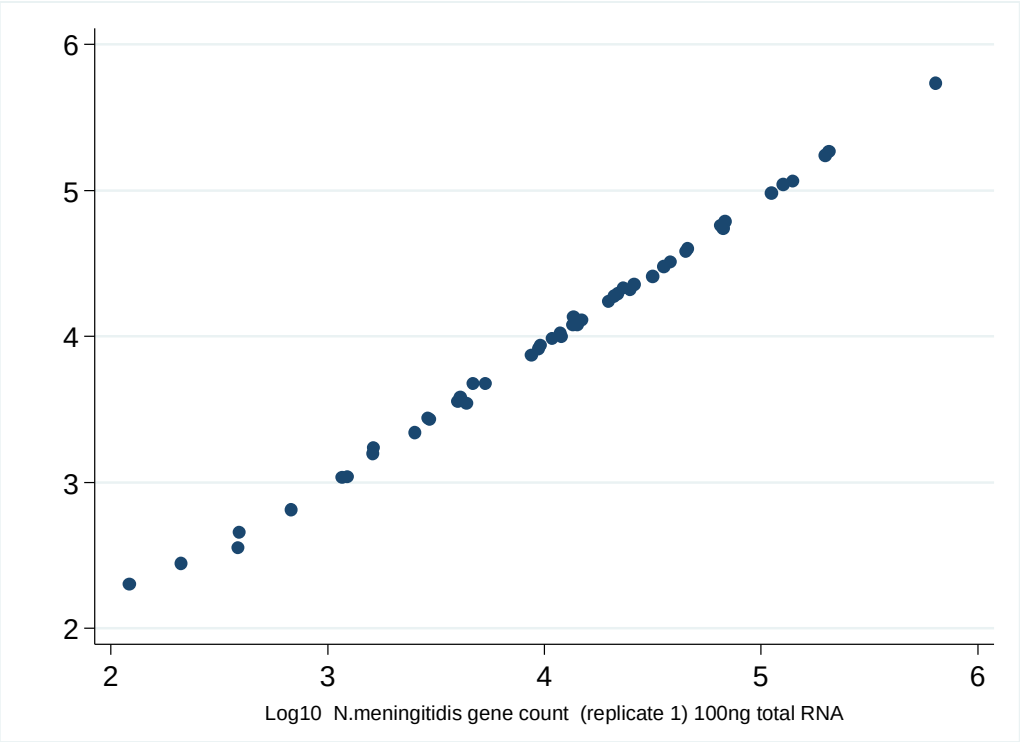


Figure 4.7 Technical reproducibility of the NanoString nCounter system for Neisseria meningitidis culture samples.

Gene transcript count of Nm from two technical replicates of 100ng RNA, Replicate 1 (x-axis) and Replicate 2 (Y-axis). The dots represent the geometric mean normalised counts (1)

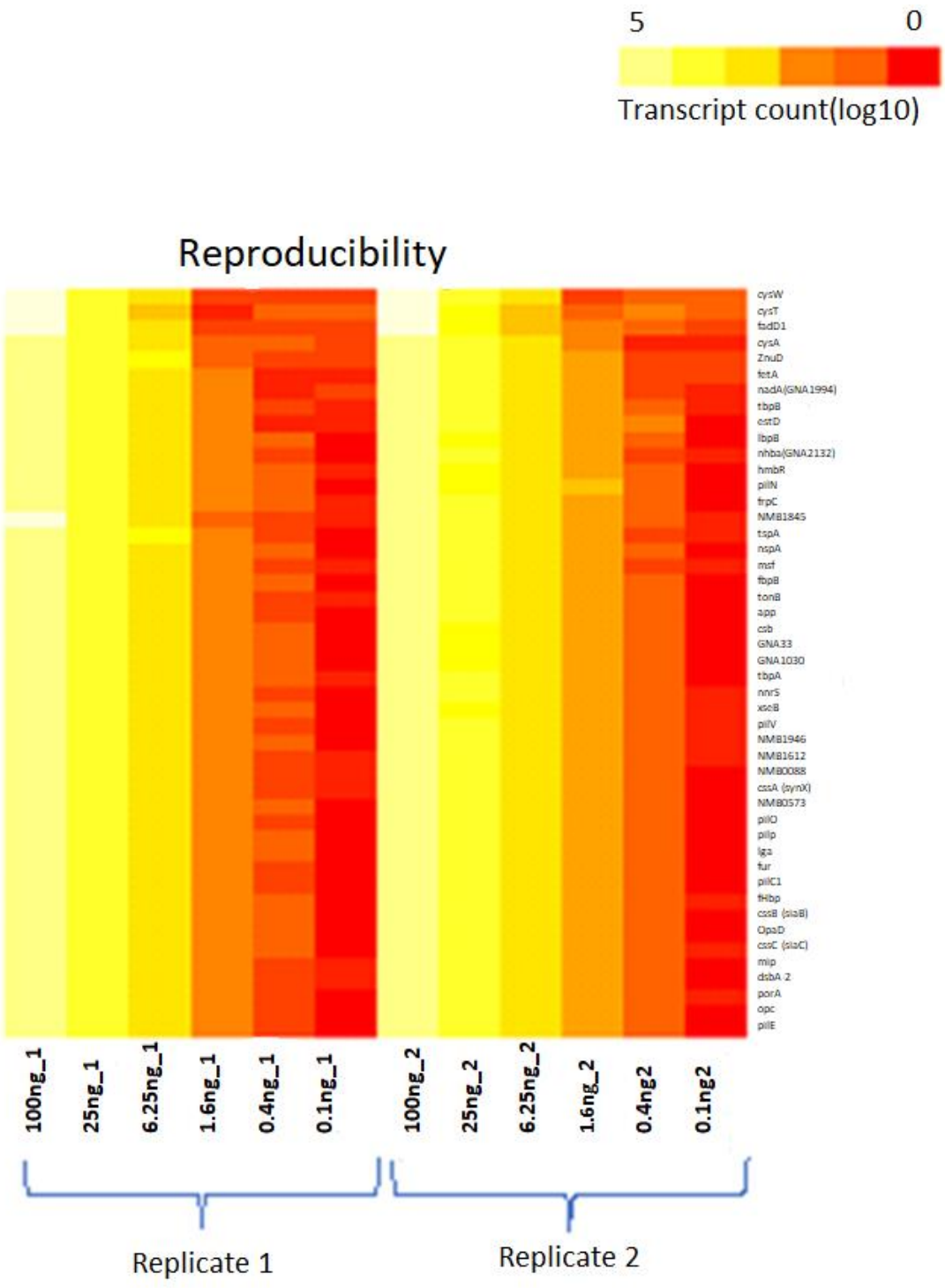


Figure 4.8 Heat map showing technical reproducibility of the nCounter platform between two replicates assayed independently (Replicates 1 and 2)

4.5.10 Nm gene detection

Ability of the NanoString custom CodeSets to detect Nm genes was assessed. Transcript counts were obtained for all 47 genes. The genes were detected from different concentrations of Nm culture isolates. Samples assayed from 100ng total RNA showed signal saturation since the transcript quantity in those samples was greater than the upper limit of detection by the NanoString instrument. However, samples which were assayed from as low as 0.1ng total RNA yielded gene counts for a few genes (Figure 4.8).

4.5.11 Fold change in gene expression

The performance of the nCounter system was assessed by measuring a change in gene expression under three different environmental conditions. A range of log2 fold changes in expression of the 47 gene transcripts (up to six-fold) was observed after exposure to high and low ambient temperatures and after iron depletion (F**Error! Reference source not found.**)

After cold shock, 30% of the genes showed up-regulation of gene expression from minimal up to six-fold change. *cysA, cysW,* and *tonB* genes showed highest up-regulation compared to gene expression in Nm cultured at 37°C (Figure 4.9A) (Table 4.6) . The genes showing most down-regulation were *tspA, znuD*, and *fadD1*. Nineteen genes showed a statistically significant (p=0.001) up or down regulation of gene expression.

A similar percentage of genes were up-regulated in the heat shock samples. *porA, dsbA* and *nadA* showed most up-regulation, whereas *tspA, cysT* and *opaD* were the

most down-regulated (**Error! Reference source not found.**B) Twenty-one genes had significant log2 fold change when compared with the control samples. (Table 4.7).

For iron depletion, *pilE, dsbA_2, NMB1612, porA, tonB, opc, NMB1646, cssA, pilO, mip* and *frbP* showed most up-regulation and *cysA, cysT, tspA, app* and *Iga* were down-regulated in the absence of iron (Figure 4.9C) (Table 4.8)All the iron regulatory genes were down-regulated after iron depletion including *fur*.

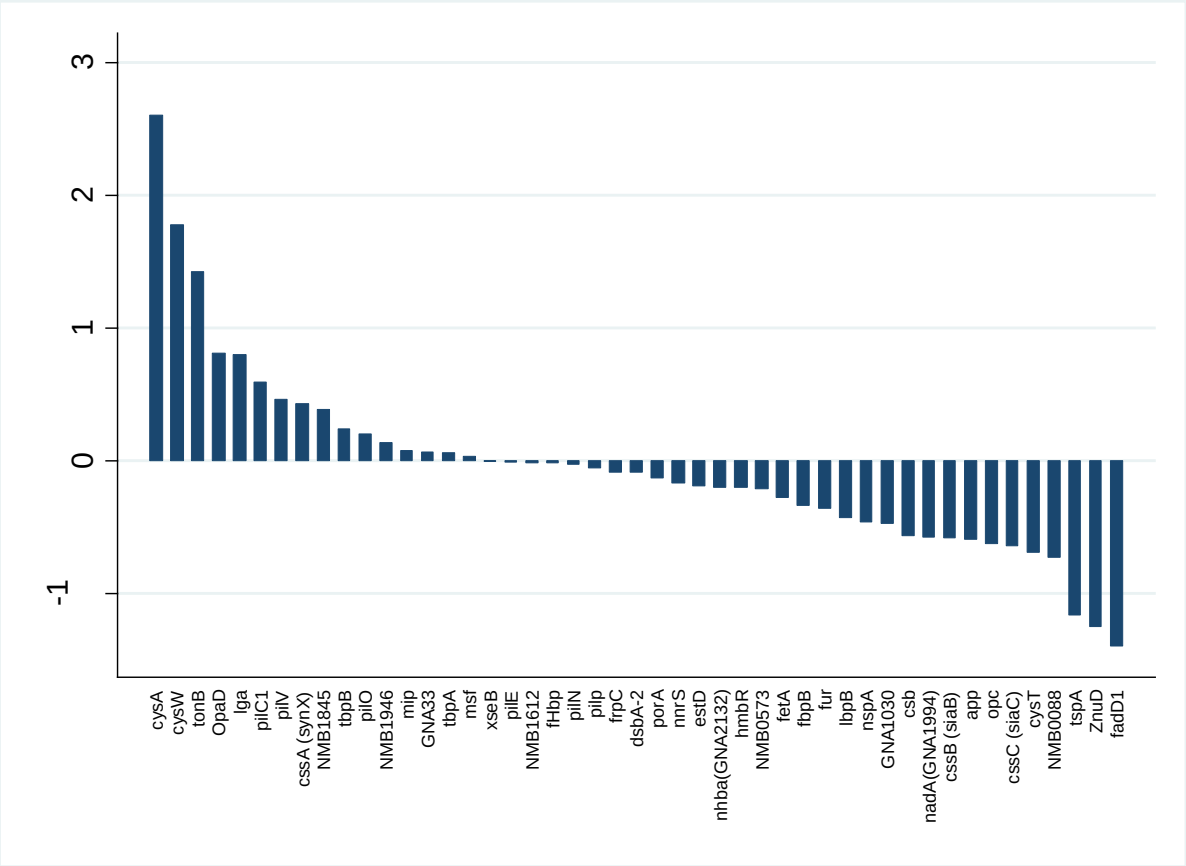


Figure 4.9 (A) Log2 fold change in gene expression across a panel of 47 Neisseria meningitidis genes induced by incubation at 26°C. The fold change is a normalised gene transcript count plotted by taking the ratio of the mean of cold exposed (26°C) gene count to the mean control (37°C) gene count.

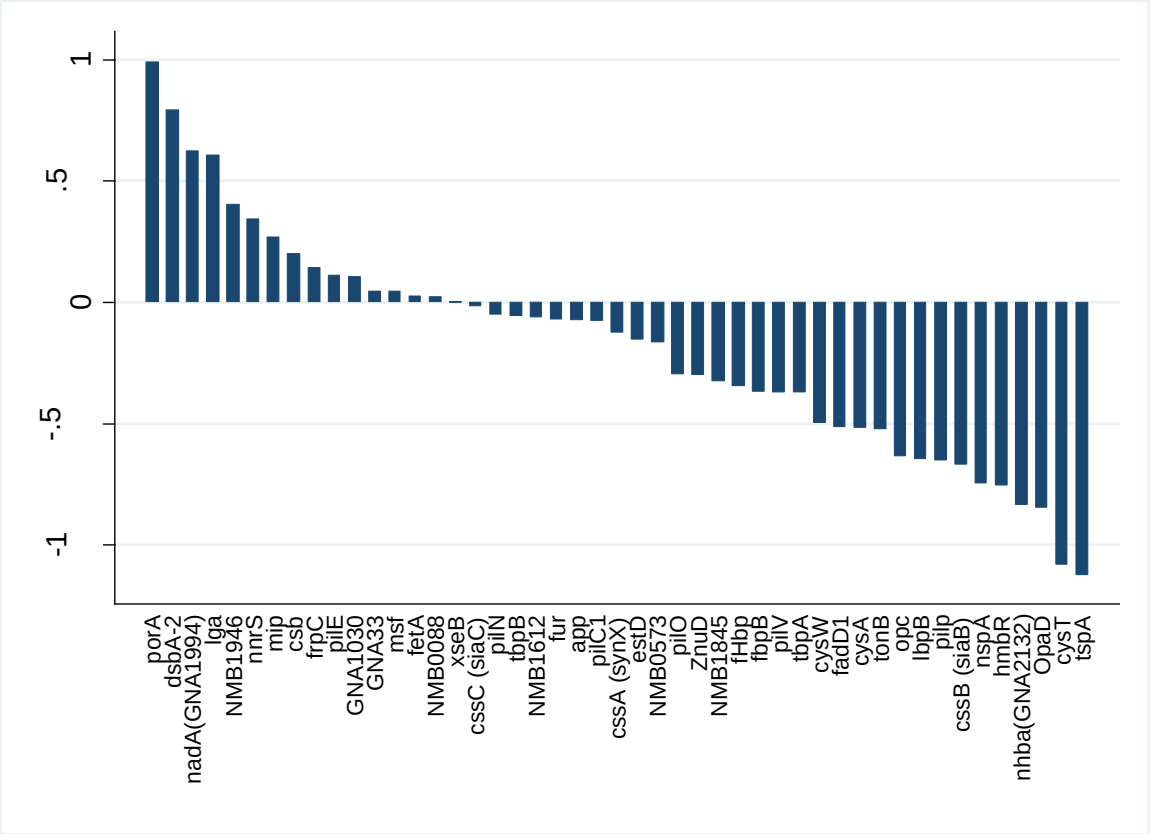


Figure 4.9 (B) Log2 fold change in gene expression across a panel of 47 Neisseria meningitidis genes induced by incubation at 40°C. The fold change is a normalised gene transcript count plotted by taking the ratio of the mean of heat exposed (40°C) gene count to the mean control gene count (37°C).

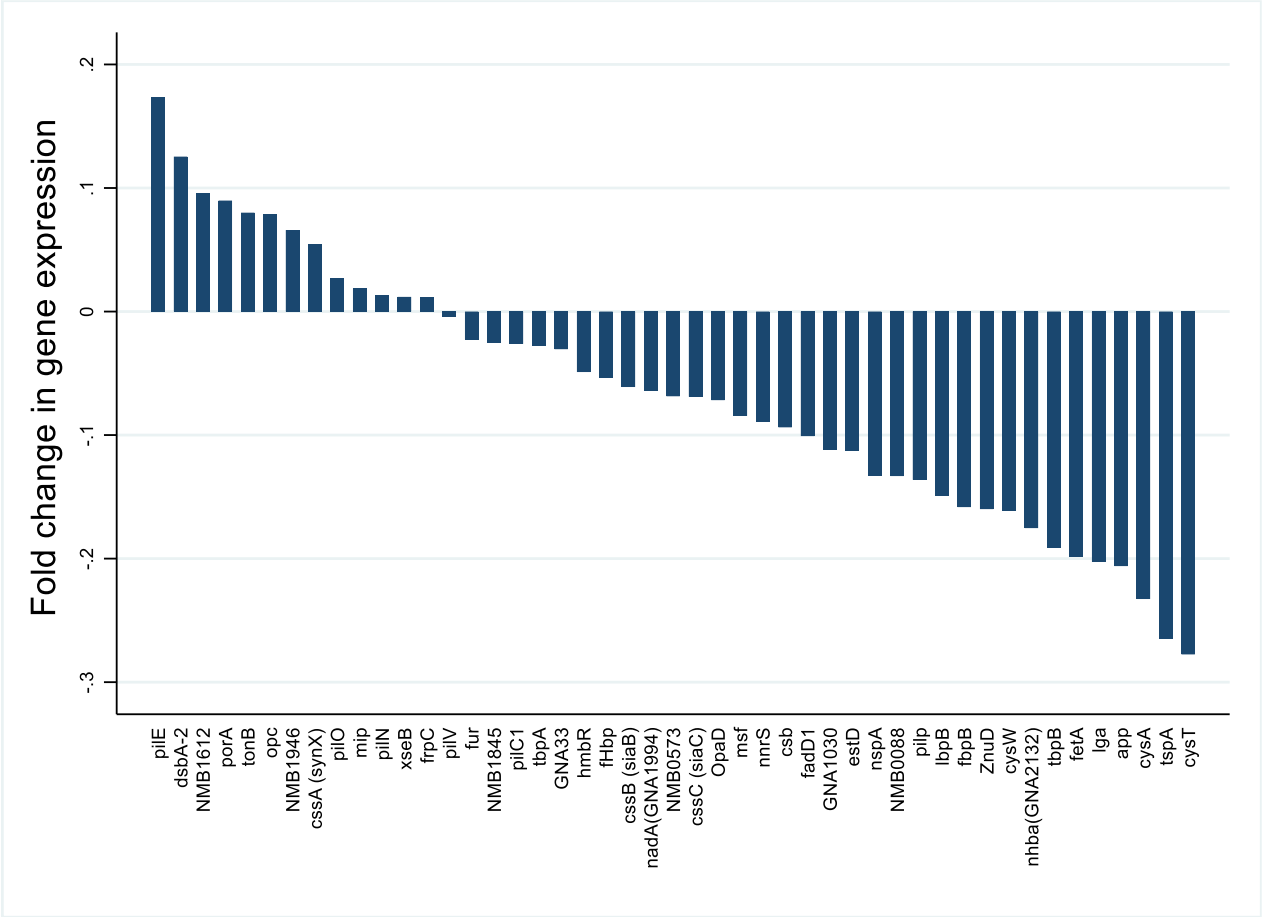


Figure 4.9 (C). Log2 fold change in gene expression across a panel of 47 Neisseria meningitidis genes induced by iron depletion. Each bar represents a different gene. The fold change is a normalised gene transcript count plotted by taking the ratio of the mean iron depleted gene count to the mean iron replete gene count.

Of the four genes relating to the novel protein vaccines (porA, fhbP, nhba, nadA) all showed down-regulation after cold shock (Table 4.6). After heat shock, porA and nadA were up-regulated' and fhbP and nhba were down-regulated (Table 4.7). After exposure to iron depletion, *porA* was up-regulated and the other three were down-regulatedTable 4.8

Table 4.6 Expression of 47 Neisseria meningitidis genes after incubation at 26°C (cold shock) compared to incubation at 37°C (control)

Gene name	Mean difference (n=6)	95% CI		Mean control (n=3)	P value
GNA1030	-3287	-4749	-1826	11755	0.001
GNA33	477	-411	1364	9995	0.292
NMB0088	-7856	-8848	-6863	19797	0.001
NMB0573	-2811	-5243	-380	20724	0.023
NMB1612	-269	-1580	1041	19393	0.687
NMB1845	1090	380	1800	3547	0.003
NMB1946	1741	234	3248	17304	0.024
opaD	44672	25962	63382	59581	0.001
znuD	-654	-1075	-233	1126	0.002
app	-2890	-4075	-1706	8491	0.001
csb	-3811	-5011	-2611	11783	0.001
cssA	6985	3425	10544	20069	0.001
cssB	-20193	-24948	-15437	60276	0.001
cssC	-37833	-48983	-26682	105111	0.001
cysA	4457	3603	5310	879	0.001
cysT	-303	-872	265	793	0.296
cysW	945	710	1180	389	0.001
dsbA-2	-8175	-28210	11860	130221	0.424
estD	-264	-793	265	2102	0.327
fadD1	-301	-508	-94	484	0.004
fbpB	-1537	-2147	-927	7260	0.001
fetA	-312	-901	277	1762	0.299
frpC	-227	-801	348	3725	0.439
fur	-8664	-12632	-4697	38647	0.001
hmbR	-422	-874	30	3155	0.067
lbpB	-697	-1222	-172	2682	0.009
iga	25651	22893	28409	34549	0.001
mip	5792	-6998	18583	108547	0.375
msf	180	-129	489	7306	0.253
nnrS	-1357	-2413	-302	11942	0.012
opc	-61431	-80681	-42182	175267	0.001
pilC1	19817	144421	25192	39239	0.001
pilE	-4693	-55407	46021	466476	0.856
pilN	-63	-218	92	2829	0.428
pilO	3654	1388	5920	24375	0.002
pilV	6668	4395	8941	17602	0.001
pilP	-1075	-4003	1853	28221	0.472
tbpA	514	-322	1351	11930	0.228
tbpB	429	-253	1111	2391	0.218
tonB	16509	21032	20986	9784	0.001
tspA	-2314	-3080	-1548	4163	0.001
xseB	-39	-1714	1637	14378	0.964
porA	-13245	-44151	17661	152732	0.401
fhbP	-736	-4087	2614	51152	0.667
nhbA	-454	-991	83	3440	0.098
nadA	-404	-670	-139	1219	0.003
tspA	-2314	-3080	-1548	4163	0.001

Table 4.7 Expression of 47 Neisseria meningitidis genes after incubation at 40°C (heat shock) compared to incubation at 37°C.

Gene name	Mean difference (n=6)	95% conf. interval		Mean control (n=3)	P value
GNA1030	-429	-1901	1022	11755	0.556
GNA34	281	-607	1168	9995	0.535
NMB0088	-391	-1384	601	19797	0.44
NMB0573	-4130	-6561	-1699	20724	0.001
NMB1612	-1698	-3008	-388	19393	0.011
NMB1845	-561	-1271	149	3547	0.121
NMB1946	4055	2548	5562	17304	0.001
opaD	-23452	-42162	-4742	59581	0.014
znuD	-152	-573	269	1126	0.479
app	-1464	-2648	-279	8491	0.015
csb	2356	1156	3556	11783	0.001
cssA	-1657	-5217	1902	20069	0.361
cssB	-21965	-26720	-17209	60276	0.001
cssC	1985	-9166	13136	105111	0.727
cysA	-216	-1070	637	879	0.619
cysT	-426	-995	142	793	0.142
cysW	-115	-350	119	389	0.336
dsbA-2	99590	79555	119625	130221	0.001
estD	-355	-883	174	2102	0.189
fadD1	-191	-398	16	484	0.071
fbpB	-1670	-2280	-1060	7260	0.001
fetA	69	-520	658	1762	0.819
frpC	-180	-754	394	3725	0.539
fur	-5119	-9086	-1151	38647	0.011
hmbR	-1208	-1660	-756	3155	0.001
lbpB	-1015	-1540	-490	2682	0.001
iga	17583	14826	20341	34549	0.001
mip	27461	14670	40252	108547	0.001
msf	168	-141	477	7306	0.287
nnrS	3659	2604	4715	11942	0.001
opc	-70307	-89556	-51057	175267	0.001
pilC2	-741	-6116	4634	39239	0.787
pilE	9690	-41024	60404	466476	0.708
pilN	-122	-277	34	2829	0.125
pilO	-4784	-7050	-2518	24375	0.001
pilV	-3510	-5784	-1236	17602	0.002
pilP	-10437	-13366	-7509	28221	0.001
tbpA	-3109	-3946	-2273	11930	0.001
tbpB	13	-669	695	2391	0.971
tonB	-2297	-6774	2180	9784	0.315
tspA	-2566	-3332	-1800	4163	0.001
xseB	1052	-624	2727	14378	0.218
porA	96803	65897	127710	152732	0.001
fhbP	-12401	-15752	-9051	51152	0.001
nhbA	-1417	-1954	-880	3440	0.001
nadA	712	447	977	1219	0.001
tspA	-2566	-3332	-1800	4163	0.001

Table 4.8 Expression of 47 Neisseria meningitidis genes after iron depletion compared to controls

Gene name	Mean difference (n=6)	95% conf. interval		Mean control (n=6)	P value
GNA1030	-567	-1139	4	5072	0.052
GNA33	-81	-734	571	2659	0.808
NMB0088	-758	-1151	-366	5697	0.001
NMB0573	-314	-702	75	4572	0.113
NMB1612	1091	-874	3056	11369	0.277
NMB1845	-158	-909	592	6304	0.679
NMB1946	620	-489	1729	9398	0.273
opaD	-1359	-2961	243	18948	0.096
znuD	-143	-488	203	892	0.419
app	-494	-784	-205	2400	0.001
csb	-111	-536	315	1184	0.609
cssA (synX)	429	-111	968	7847	0.12
cssB (siaB)	-564	-1011	-117	9234	0.013
cssC (siaC)	-437	-1900	1026	6336	0.558
cysA	-183	-592	227	785	0.382
cysT	-131	-404	141	473	0.345
cysW	-66	-205	73	411	0.351
dsbA-2	7232	-890	15354	57671	0.081
estD	-195	-460	69	1732	0.148
fhbP	-510	-774	-246	9548	0.001
fadD1	-27	-144	91	264	0.657
fbpB	-329	-790	131	2080	0.161
fetA	-184	-5564	196	927	0.342
frpC	35	-277	347	2966	0.828
fur	-639	-2859	1580	28296	0.572
hmbR	-59	-376	258	1210	0.714
lbpB	-118	-473	236	794	0.512
iga	-1090	-2402	222	5379	0.104
mip	1259	-2308	4825	66547	0.489
msf	-445	-897	7	5266	0.054
nadA	-37	-124	50	577	0.404
nhba	-358	-634	-82	2042	0.011
nnrS	-1645	-4619	1330	18484	0.279
nspA	-65	-264	134	491	0.52
opc	3198	-1058	7453	40690	0.141
pilC1	-476	-1641	690	18176	0.424
pilE	45681	21617	69745	263266	0.001
pilN	24	-65	114	1856	0.594
pilO	234	-687	1155	8685	0.619
pilV	-43	-1008	922	10448	0.93
pilP	-827	-2192	538	6069	0.235
porA	3889	-909	8686	43294	0.112
tbpA	-109	-279	61	3936	0.209
tbpB	-201	-635	233	1051	0.364
tonB	749	-523	2021	96363	0.248
tspA	-362	-707	-17	1368	0.04
xseB	144	-838	1126	12134	0.774

4.5.12 Nm-negative pharyngeal samples, probe validation

To assess specificity of the probes, gene expression was measured in Nm negative samples. Out of the 31 Nm specific genes, one was found to have a high gene count (*dsbA_2*) in six of the twelve Nm-negative pharyngeal samples. All the other genes had either a zero count or were below the maximum internal negative control count. The result confirms that nearly all gene counts detected from the *SodC* Nm positive samples are true gene counts.

4.6 Discussion

This study showed for the first time that mRNA can be successfully detected and quantified from Nm cultures using custom designed CodeSets for a digital multiplexed gene detection system, without the need to amplify the genes, and from very small quantities of total RNA. The technique showed high reproducibility in technical replicates assayed independently. Sensitivity, reproducibility and ability to detect fold change using this novel technique were established.

The RNeasy technique was easier to perform, gave more reproducible results, and delivered a higher quality and quantity of RNA than the acid:phenol:chloroform method. The acid:phenol:chloroform method is less expensive, but (i) it uses hazardous chemicals and (ii) the need to change to different size tubes between the first and second extractions may reduce quality and quantity of the RNA. The RNeasy method is more expensive, but easier to perform without the need for special equipment or potential exposure to hazardous reagents. Both methods take equivalent hands-on time to extract the RNA even though the acid:phenol:chloroform technique

needs overnight incubation. Although the initial results from the acid:phenol:chloroform technique suggested that much higher quantities of RNA had been obtained, RNA quality was low, and the results were highly variable. After clean-up, RNA quantity dropped considerably, suggesting that the initially high levels were due to contamination. As the RNeasy technique also gave higher gene expression counts than from crude lysate without RNA extraction, it was selected as the extraction method for the subsequent experiments. In addition, the processing time from sampling to storage in RNAlater was subsequently shortened, which improved the RNA yield while maintaining high quality.

Forty-seven genes were selected for study based on actual or potential vaccine antigens or proteins important in colonisation and metabolism of Nm. Some of these genes (n=16) were highly conserved among other neisserial species, so it was not possible to design a fully species-specific Nm probe for these. Among the other 31 genes, zero counts were validated for 30 genes against PCR confirmed Nm negative samples. Cross-reactivity would be expected to have an impact when processing samples from non-sterile sites such as pharyngeal samples, where other commensal neisseria species may be present. This is explored by WGS in Chapter 5. Where the probe is not specific for a particular gene, other methods such as RNA-Seq would be needed to confirm expression. For multi-copy genes, as the probes were targeted at conserved regions, a limitation is that it may be difficult to identify which copies are transcribed. Although the reliability of the NanoString platform has been extensively reported for other organisms (102, 103, 111, 114, 140), ideally the results should be cross-validated with other methods such as reverse transcriptase qPCR for selected genes in subsequent experiments (see Chapter 4).

Although this study was intended to assess the method and not specifically to identify which genes were expressed under different conditions, a range of gene expression was successfully detected. The up/down regulation and fold change in gene expression of the selected 47 genes was shown for samples cultured under different environmental and nutritional stress conditions. Guckenberger et al (124), who used micro-arrays to measure Nm gene expression after heat shock at 45°C, had results consistent with those in our study, including for example, up-regulation of *dsbA_2* and down-regulation of *pilO* and genes involved in ABC transport system (*cysA, cysW, cysT* in our panel). In contrast, two of these genes (*cysA, cysW*) were up-regulated on cold exposure in my study, *cysA* gene expression increased approximately six-fold in response to Nm incubation at 26°C.

Iron is a vital element for growth and survival of Nm (141, 142). After Nm was cultured in iron-depleted conditions, up and down regulation of genes in response to this iron stress was seen. Most of the sulphate ABC transporter genes from the panel of genes such as *cysT* and *cysA* were down-regulated, as also reported in a study by Mellin (125). *fur*, an important gene for ferric uptake regulation that prevents gene expression initiation in an iron-replete environment (125), showed little evidence of down regulation in response to iron depletion but exposure was of short duration (15 minutes) and longer term exposure to these conditions may be needed before changes in expression of this gene are observed. The cys genes responded quickly to stress conditions, possibly related to their importance in colonisation, and they provide an example of proof of principle of the NanoString technique where down-regulation of cys genes in response to heat and to iron depletion are both consistent with previous research.

A better understanding of Nm gene activities in meningococcal carriers will give insights as to whether the bacteria could be eliminated from carriage by specific protein vaccines and as to which genes are important for the survival of the pathogen in a normal commensal state. Much research has been done to study *in vitro* Nm gene expression of potential vaccine candidates such as those in the Bexsero vaccine. However, little is known about *in vivo* gene expression, which has relied on indirect evidence from animal studies. Application of this technique to Nm pharyngeal samples is expected to be challenging as RNA concentrations in such samples will be relatively low, variation in levels of gene expression is wide, and RNA from other pharyngeal bacteria is likely to be present. This is explored in the following chapter.

Chapter 5. Gene expression profiling of *Neisseria meningitidis* from pharyngeal carriage samples and its association with carriage density

5.1 Introduction

Gene expression profiling is a powerful tool for studying the activity of a cell or cellular function by measuring the pattern and quantity of mRNA in a given sample. For Nm, gene profiling has the potential to increase understanding of genes involved in meningococcal colonisation and invasion (143-145). Pharyngeal carriage of the bacterium is the first critical step leading to infection and potentially to disease (146, 147). Several genes are involved in the attachment of Nm to the endothelial cells and establishment of carriage (143, 146). Studying the expression level of those genes may contribute to understanding which genes are more actively involved (145).

It is also important to assess the relationship between gene expression and bacterial density, since density in pharyngeal carriers of Nm shows extensive variation, with the majority of carriers having relatively low density ($<10^3$ gene copies/ml) (65). Transcriptomic analysis of the levels of expression of Nm genes in pharyngeal swab samples may help to predict the impact of meningococcal candidate vaccine protein antigens on transmission.

Most gene expression studies of Nm have previously been done either in laboratory-cultured samples, by mimicking the internal environment of human host, or in cell line experiments (93, 143, 148). Gene expression patterns of Nm from *in vivo* pharyngeal carriage samples is challenging due to the presence of host mucosal cells and multiple

bacterial species in the nasopharynx and because Nm is only present at low density in most pharyngeal carriers. Results demonstrating the reproducibility and sensitivity of detecting Nm genes directly from culture samples are shown in Chapter 4 using the NanoString nCounter system (1). In this chapter, work describing application of this method to Nm to transcript detection in complex pharyngeal samples is presented. Cross validation of the NanoString transcript count results will also be validated for one gene *(fhbP)* using RT-qPCR.

5.2 Aim

To examine the expression pattern of 47 Nm genes from pharyngeal carriage samples collected from healthy carriers and evaluate the association between expression and Nm carriage density.

5.3 Objectives

- To extract total RNA from Nm positive pharyngeal carriage samples
- To profile 47 selected Nm genes using the NanoString nCounter system
- To assess the technical reproducibility of the NanoString platform for Nm pharyngeal samples
- To compare Nm gene expression in different bacterial carriage densities
- To assess the association between Nm gene expression and carriage density
- To compare the pattern of gene expression between three carriage studies
- To cross-validate the NanoString transcript count for *fhbP* with RT-qPCR
- To assess gene expression pattern within individuals from BrisMenNHC longitudinal samples

5.4 Methods

5.4.1 Pharyngeal swab sample collection, transport and storage

A total of 3643 pharyngeal swab samples were collected in the BrisMenNHC study from Bristol students aged 16-19 years in up to five monthly visits (Appendix D) using a double headed swab. One head of the swab was put into RNA-preservation medium RNAlater and the other head into STGG. RNAlater is an RNA preservative medium, which improves the integrity and yield of RNA in the samples (3.2.13.1).

Of 267 Nm positive samples, gene expression profiling was done on samples with bacterial density >100 gene copies per ml (n=53). Samples with bacterial density >100 gene copies per ml from two other meningococcal carriage studies in teenagers, SPIT (n=19) and a Portuguese study (n=22), were also selected for gene profiling. The SPIT study swab samples were collected from students aged 16 to 18 years in Bristol (Appendix E) and the Portuguese study samples were collected as stated previously (149), using the same methodology as BrisMenNHC. For the Portuguese study, STGG prepared in the UoB was sent to Portugal. The Portuguese samples were transported to the UoB using dry ice. For all three studies, bacterial identification by qPCR using *sodC* gene and carriage density measurement was done in Bristol by the Finn laboratory group. The *sodC* qPCR was done using a protocol published in 2016 (65), explained briefly in 3.2.17.

5.4.2 RNA extraction from pharyngeal swab samples and DNA digestion

Total RNA was extracted from pharyngeal samples stored in RNAlater and purified using RNeasy mini kit (Qiagen, 76506) following the manufacturer's protocol as explained in Chapter (3.2.5). Both on and off-column DNA digestion was performed

using RNase-free DNase set (Qiagen) and TURBO DNase respectively (3.2.6 and 3.2.7). The quality and quantity of the total RNA was measured using a NanoDrop Denovix D-11+ Spectrophotometer (3.2.14.1).

5.4.3 NanoString nCounter system

The nCounter assays for the pharyngeal swab samples were done using a SPRINT profiler (3.2.15.1).

5.4.4 NanoString probe design and hybridisation

The probe design for the custom CodeSet is explained in Chapter 4 (4.4.2). Forty-seven different genes were profiled from each sample simultaneously using NanoString XT formulation following the manufacturer protocol using Nm custom CodeSets (Nm_YKT01)(1) (3.2.15). The total RNA samples extracted from the pharyngeal swab samples were thawed on ice with the reporter CodeSet and capture probe set reagents before hybridisation (3.2.15.2).

5.4.5 Validation with RT-qPCR

In order to validate the NanoString nCounter transcript count for the *fhbP* gene, a RT-qPCR was performed (3.2.16). RT-qPCR is a technique that is commonly used for gene expression assays. It allows the amplification of cDNA, which is reverse transcribed from RNA using a reverse transcriptase enzyme. The cDNA is then used as a template to do RT-qPCR. As the sample volumes were small, a two-step RT-qPCR was done because of the higher sensitivity and ability to test more than one target. The two-step RT-qPCR involves changing RNA to cDNA by reverse transcription followed by qPCR (Figure 5.1).

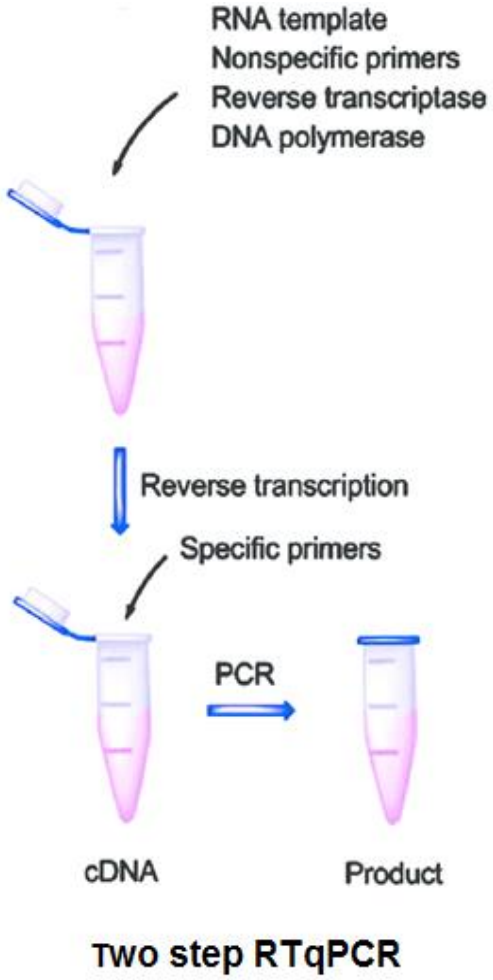


Figure 5.1 The steps for the two step RT-qPCR

The crucial step in RT-qPCR is to make sure that the RNA samples contain no DNA to avoid false positive results. After DNA digestion using on-column Qiagen DNase, the 48 total RNA samples were tested for the presence of genomic DNA using *sodC* PCR and half of the samples were found to have gDNA contamination. Further DNA digestion off-column using TURBO DNase was done to remove any remaining gDNA present in the *sodC* PCR positive samples (3.2.7). The double digested total RNA was then checked again for the presence of gDNA and all samples were found to be free of DNA. The clean total RNA was then dry ice shipped to the University of Leicester where the RT-qPCR was done. The total RNA was thawed on ice at room temperature

and reverse transcribed to cDNA using reverse transcriptase (RT) in the presence of oligo-dNTPs and random hexamers using a protocol developed by University of Leicester (3.2.16). A stable cDNA was amplified using Fast SYBR green. Two separate PCR reactions for (i) Variant 1/ subfamily A and (ii) Variant 2/3 / subfamily B was made. Housekeeping gene *gdh* was used as an internal control assay protocol.

The expression of fHbp variants was tested in 48 of the 53 Nm positive samples that previously had been tested with the NanoString SPRINT profiler. Five samples were not tested as expression had not been completed at the time of the sample shipment to the University of Leicester. All the samples were run in triplicate for RT-qPCR. A standard was prepared and used as a calibrator. The standard was synthetic oligonucleotides prepared from known gene sequences for the two sets of variants, using H44176(154) for Variant 1/subfamily B and 240588(138) for Variant 2/3/subfamily A. The standard was prepared to cover the lowest and highest dilution, from $6.16x10^4$ copies/µl - 6.16x100 copies/µl. A calibrator was used for each variant 1 and variant 2/3 (4 and 138 respectively). The standards and the samples were assayed in parallel on the same plate. The reaction mixture was done in a 96 well microamp fast optical reaction plate. Once all the reaction mixture was in the plate, it was sealed with a MicroAmp adhesive film to avoid evaporation. Primers, Probes and real time programme for the fHbp variant tests are listed in (3.2.16).

5.4.6 Checking for the presence of genomic(g)DNA in the RNA samples

Genomic DNA (gDNA) contamination of RNA samples obtained from the pharyngeal samples can lead to inaccurate measurement of gene expression using RT-PCR but will not affect NanoString analysis since the hybridisation temperature does not reach denaturation temperature. A qPCR was used to detect gDNA in the RNA samples

using the *sodC* gene. 48 BrisMenNHC RNA samples which were ready to take to the University of Leicester at the time were checked for the presence of contaminating DNA in the total RNA samples. The assay followed a *sodC* protocol as explained in (3.2.17). The stored RNA samples were thawed on ice and spun briefly. A master mix was prepared using a QIAagility pipetting robot to pipette all the reagents: a fast-universal mix(10µl), primer pairs(300nM) and probes (100nM). 5µl of each sample was pipetted manually into each reaction mixture. The primer and probes used for *sodC* qPCR are listed in (3.2.17 and Table 3.6)

5.4.7 Statistical analysis

The raw gene count data were obtained from the SPRINT profiler as a count (RCC) zip file for each set of 12 samples. The RLF that contains all the probe design information was first uploaded on nSolver software in order to read the RCC files. Once the unzipped data is exported to nSolver, QC checks were done for each run using four quality measures (3.2.15.6). The geometric mean count of the negative controls was calculated and subtracted from each count. This normalised count was then used to calculate transcript count in 30µl of total RNA extracted from the RNAlater collected pharyngeal swab samples. Gene count per bacteria was calculated from the respective densities of each samples and expressed as gene count per 1000 bacteria. The final value was then transformed to its logarithmic to the base 10 value to plot the graphs and do the analysis.

5.4.7.1 Linear regression data analysis of gene expression

The relationship between gene expression, the dependent variable, and bacterial density, the independent variable, was assessed by simple linear regression using Stata version 15. The linear regression was done to model the relationship between gene expression and bacterial carriage density that could take account of other variables.

A total of 39 subjects from BrisMenNHC study who had independent observations were included in the analysis (26 had one visit only, 12 had two visits and one had three visits, making 53 samples in total). The result from the first visit was used to do the regression analysis.

Before performing linear regression, the gene expression data was checked if it meets the assumptions of regression. In order to assess the distribution of the gene expression data, the data were first visually checked by plotting a scatter plot and histogram on Stata. Since the data were positively skewed, the values were transformed to log10 values (150). Before proceeding to fit the line of regression, the log data were checked for their approximate linear association using a two-way scatter plot (supplementary table 2) and a distributional diagnostic plot (qnorm) (Figure 5.11) was done on Stata to test the normality of the log transformed data.(Figure 5.10B). I assessed the association between gene expression and bacterial density using a simple linear regression model ($Yi= \beta0+ \beta1Xi+ei$) for each of the genes.

Gene expression= β0+ β1 bacterial density + ei

β0 and β1 are regression coefficients which are unknown parameters to be estimated.

β0 = intercept(constant) and β1 = slope or the coefficient of “X” and e is the residual.

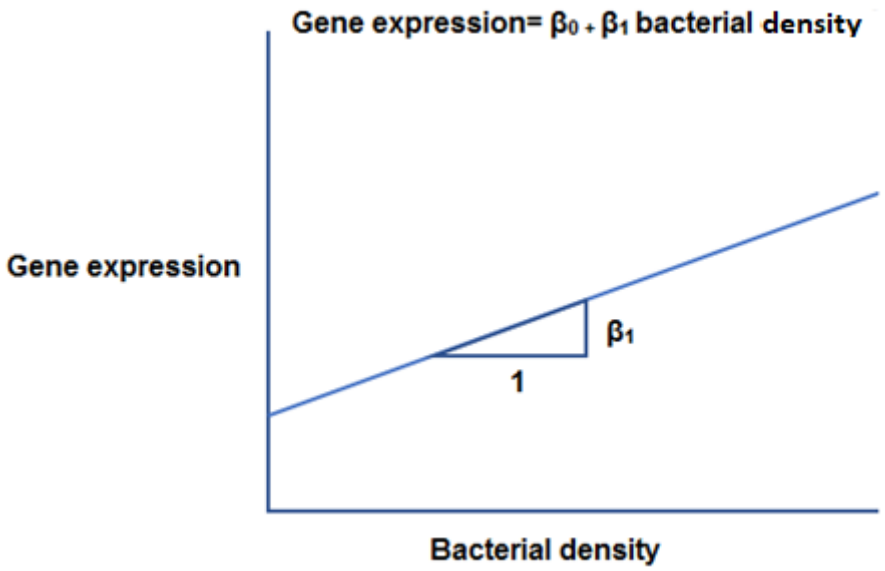


Figure 5.2 The intercept and slope of the regression equation,

gene expression= B0 +B1 bacterial density, the intercept B0 is the point where the line crosses the y-axis and gives the value of Y for x=0. The slope B1 is the increase in Y corresponding to a unit in X.

5.4.7.2 Spearman's rank correlation

Spearman rank correlation was also used to assess the association between gene expression and bacterial carriage density. Spearman rank correlation test is a non-parametric test which measures the association between ranked variables. A Spearman rank correlation was done to assess the strength and direction of the monotonic relationship between gene expression and bacterial carriage density. Below is an example of a monotonic relationship.

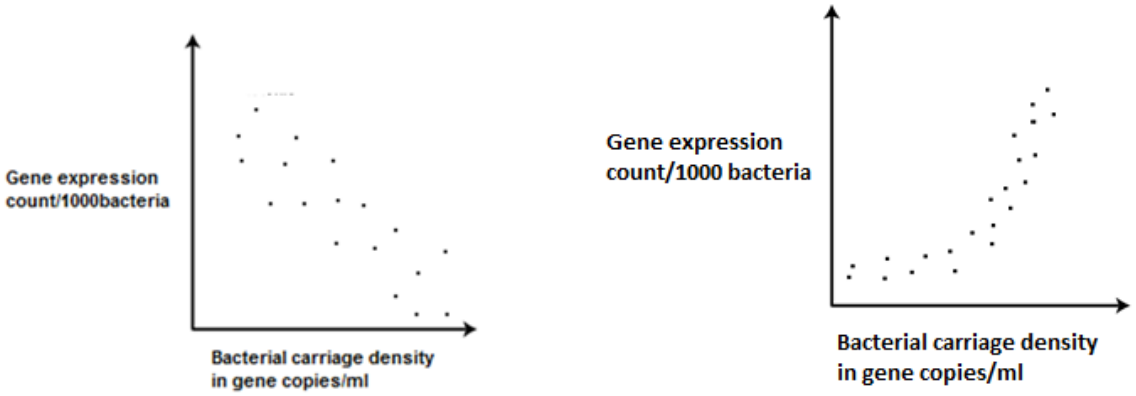


Figure 5.3 Example showing the monotonic relationship of gene expression and bacterial carriage density.

5.4.8 Trend of longitudinal BrisMenNHC samples

Thirteen paired longitudinal pharyngeal swab samples from different visits were examined to assess trends of gene expression between the samples obtained at different visits for each gene and subject. The trend for those samples was plotted using paired samples using Stata 15 and assessed visually.

5.4.9 Cohen's Kappa

Cohen's Kappa analysis was done to calculate the inter-rater agreement between NanoString transcript counts for *fhbP* compared with RT-qPCR

Cohen's weighted kappa analysis was done to compare the agreement of gene expression pattern of Nm in three carriage studies (BrisMenNHC, SPIT and

Portuguese study). The percent agreement and k (kappa) were tested using Stata software after ordering expression from 1-47. The P values were also calculated.

5.5 Results

5.5.1 Density distribution of carriage in BrisMenNHC Nm-positive samples

Samples suitable for gene expression analysis were identified through measurement of carriage density. The density distribution of samples from carriers were skewed to the right with most carriers having low numbers of gene copies/ml (Figure 5.4). Of 267 Nm positive samples from 5 visits for which consent had been given for further analysis, 56 samples had sufficient density (>100 gene copies/ ml) for gene expression analysis. whereas genes with less than 100 gene copies per ml had no measurable gene count and were excluded from the analysis. Out of the 56 samples, gene expression was measured in 53 samples. Three samples were excluded because consent was not obtained for further DNA analysis. Those with bacterial carriage density log value >=3 were termed high density and those with a carriage density log count 2 to <3 were termed medium density (65). Density in these samples ranged from 106 - 22,852 gene copies per ml.

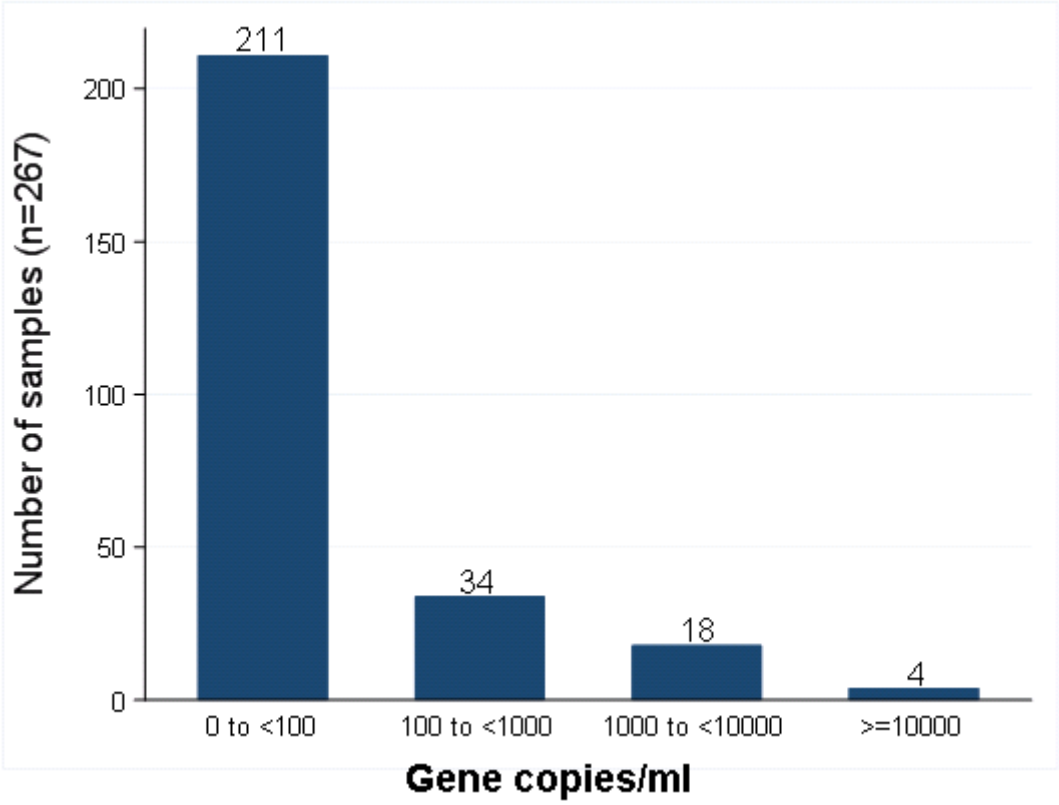


Figure 5.4 Bacterial density distribution of Neisseria meningitidis positive samples from carriers expressed in gene copies per ml.

The x-axis is the number of samples and the y-axis is the bacterial carriage density expressed in gene copies/ml in each category. The total number of samples is 267.

5.5.2 Demographic characteristics of BrisMenNHC Nm-positive subjects

To describe the demography of the study subjects, their characteristics are shown in Table 5.1. There were 25 male and 14 female students from 7 different schools in Bristol. 87% of the students were from the white ethnic group, mostly (61%) aged 17 years (n=24).

Table 5.1 Demographic factors of Neisseria meningitidis positive BrisMenNHC subjects, N= 39

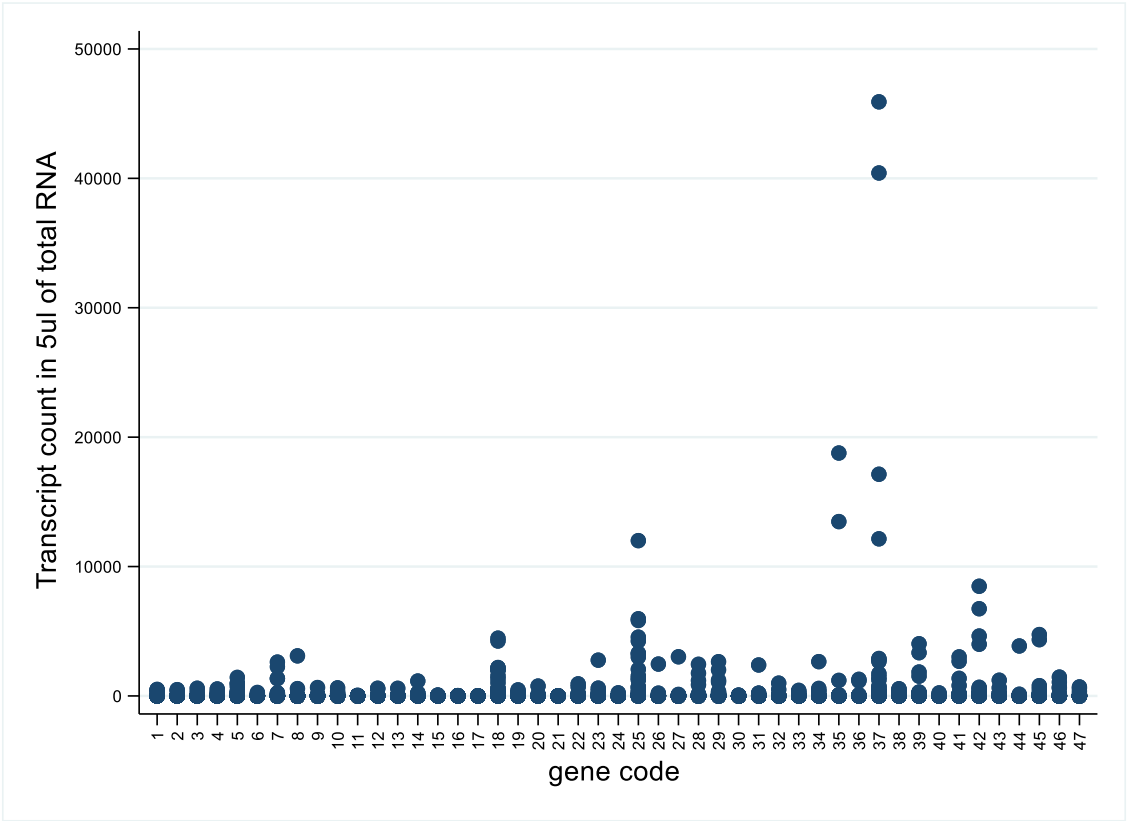
Variables	Total number of observations	Percent
Gender		
Male	25	64.1
Female	14	35.9
School year		
year 12	16	41.0
year 13	22	56.4
Other	1	2.6
Ethnicity		
White	34	87.2
Asian	3	7.7
Black	1	2.6
Mixed/other	1	2.6
Age group		
15/16	8	20.5
17	24	61.5
18	6	15.4
19	1	2.6
URTI symptoms at time of sampling		
No	26	66.7
Yes	13	33.3
Antibiotics		
No	35	89.7
Stopped in last month	3	7.7
Currently taking	1	2.6
Cigarette smoking		
No	38	97.4
Yes	1	2.6
Other smoker at home		
No	32	82.1
Yes, outside	7	18.0
Visits to pub in last week		
0	24	61.5
1	9	23.1
2 to 7	6	15.4
Number of boys/ girls kissed in last week		
0	22	56.4
1	17	44.0

5.5.3 Quantity of the total RNA from pharyngeal swab samples

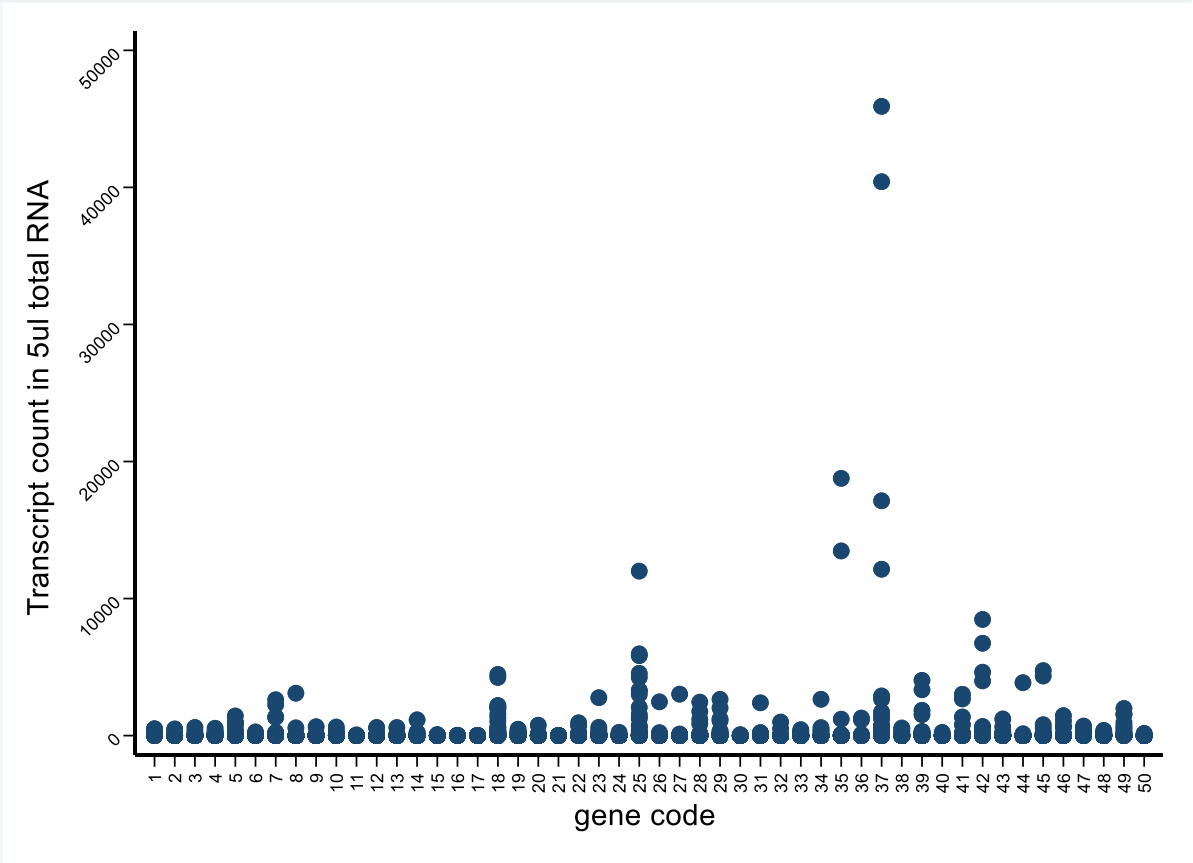
To document RNA quantity among the samples, the total RNA for each sample was measured. The 53 pharyngeal samples had total RNA quantities ranged from 1-31ng/µl measured using nanodrop (3.2.14.1).

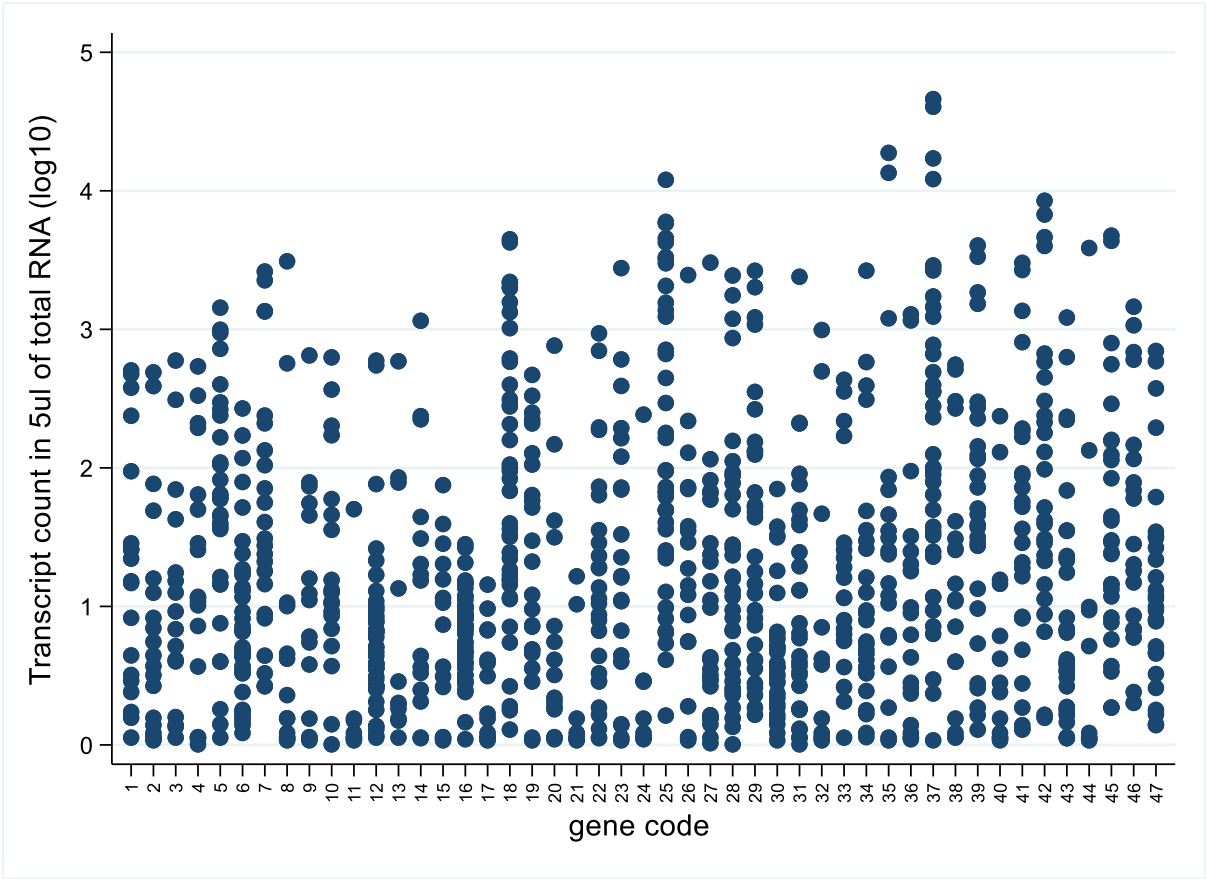
5.5.4 The range of Nm transcript detection

Numbers of transcript counts per gene by sample were documented to show the extent of variation in expression among individual samples. The expression showed an extensive range of mRNA transcript counts from 0 to 45,921 transcript count in 5µl total RNA (Figure 5.5). Transcript counts of zero after normalisation were taken as “not expressed” or “turned off”, transcript counts >=1 were taken as “expressed”. This count was then adjusted to the density of samples and expressed in gene copies per 1000 bacteria to do further analysis.


Transcript count in 5ul of total RNA
50000
40000
30000
20000
10000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47
gene code

A.


Transcript count in 5ul total RNA
50000
40000
30000
20000
10000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
gene code


Transcript count in 5ul of total RNA (log10)
0
1
2
3
4
5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47
gene code

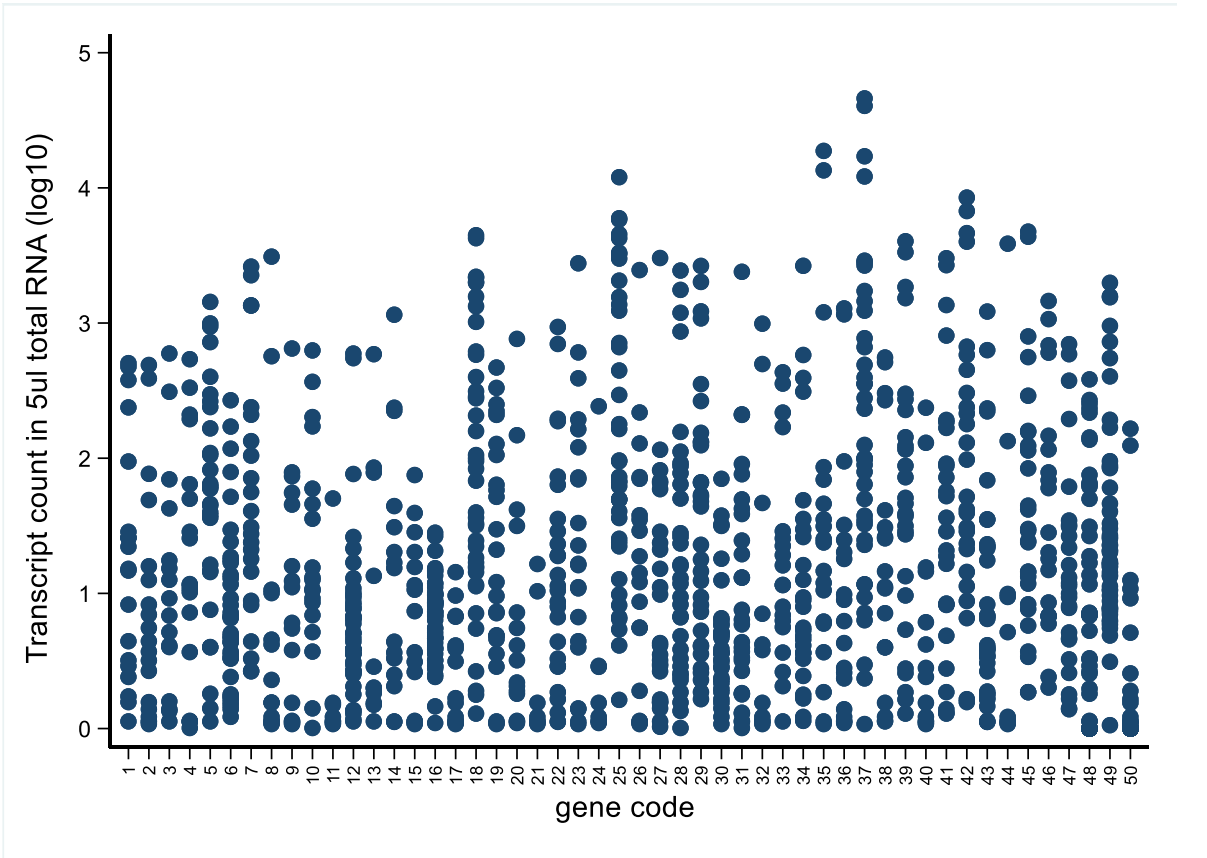

Transcript count in 5ul total RNA (log10)
0
1
2
3
4
5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
gene code

Figure 5.5 Transcript count of Neisseria meningitidis in 5ul of total RNA after negative control normalisation.

The blue dots indicate individual gene counts (A. untransformed count B. log10 transformed counts).

5.5.5 The range of Nm transcript count

Mean expression per gene was compared to show the overall extent of variation in expression for each gene. Of the 53 samples, 12 were from two repeat visits for the same individuals. One individual had three visits and 26 individuals had single visits. The analyses comparing gene expression were restricted to results of 39 independent samples i.e. the first visit from those who had more than one visit.

Twenty-two genes were expressed in more than half of the samples, one (*cysT*) was detected in all 39 samples. *fur, pilE, dsbA_2, opc* and *nadA* had the highest mean gene expression (>3000 transcript counts/ 1,000 bacteria) whereas *fadD1, csbA, cysW, frpC , pilV, nhbA, gna33* and *fhbP* had the lowest mean expression (<50 gene counts/1000 bacteria) (Figure 5.6, Figure 5.7). Most carriers had a low transcript count as indicated in the density of count in the heatmap (Figure 5.7).

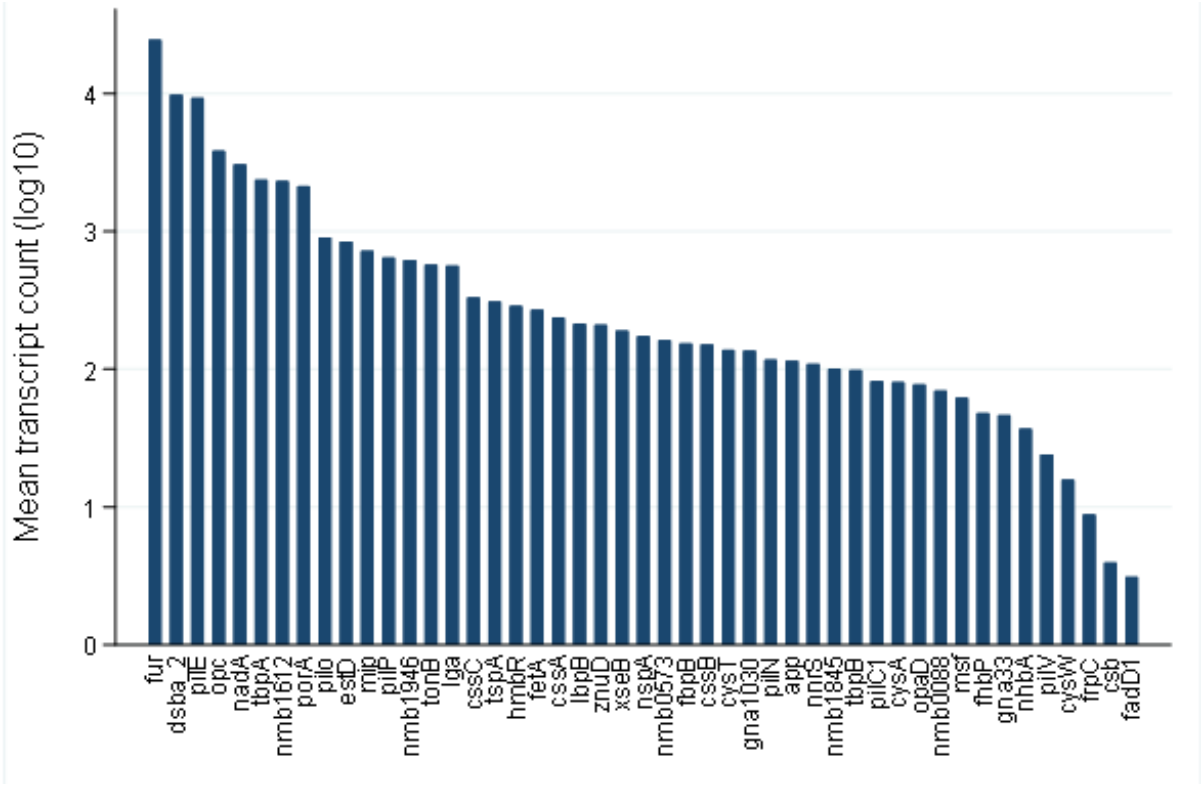


Figure 5.6 Mean transcript count of Neisseria meningitidis 47 genes (N=39).

The x-axis shows the 47 genes and the y-axis shows mean of the transcript count/1000 bacteria (log10) as a measure of gene expression. The mean was calculated after subtracting the background (negative control counts) from the Nm gene counts.

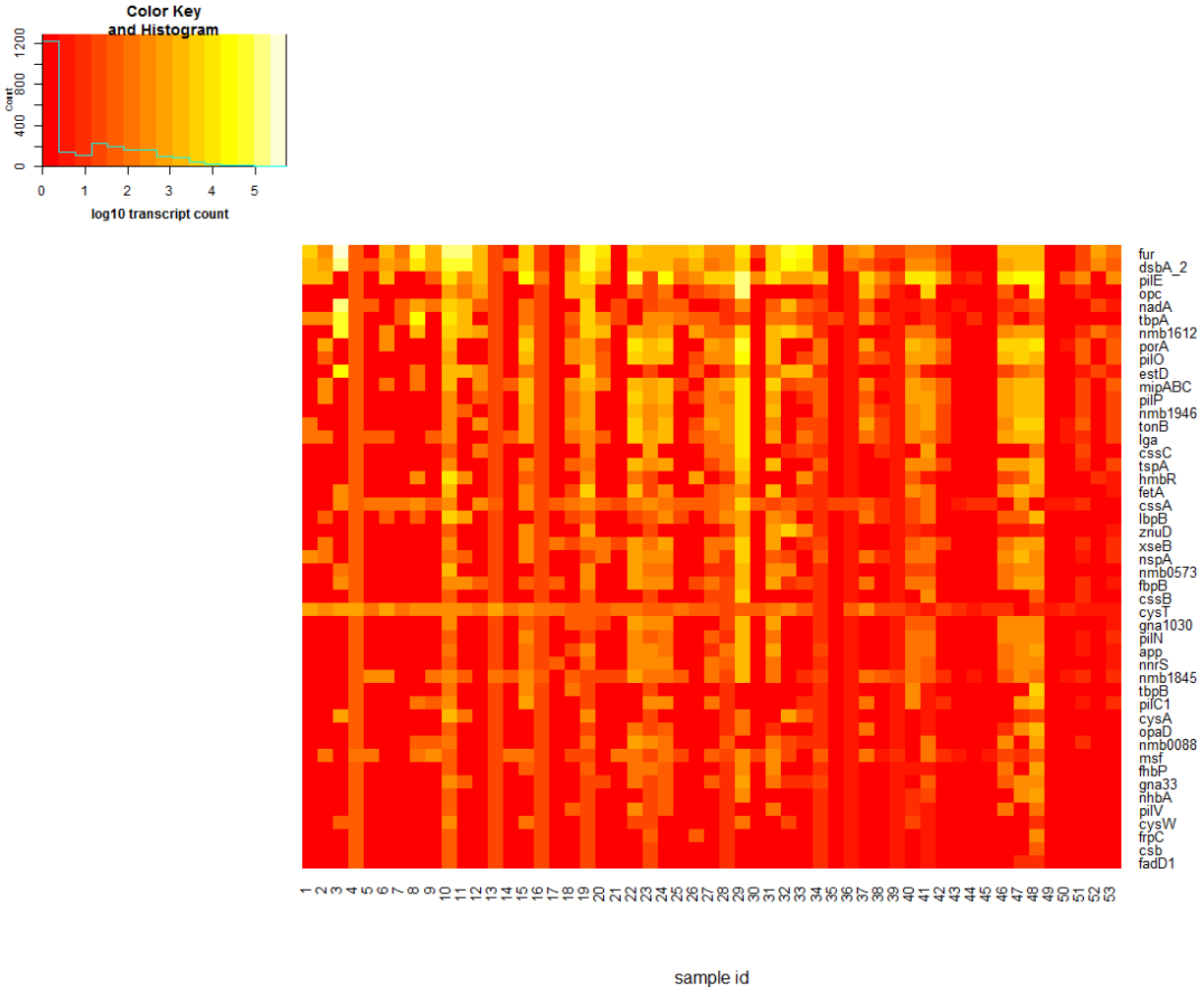


Figure 5.7 This heatmap shows the gene expression patterns of 47 genes in 53 Neisseria meningitidis-positive samples. The values indicate the normalised gene transcript count/1000 bacteria (log10). The transcript count is depicted using different colours. The bright red colour shows a low transcript count while yellow or white represent a high transcript count.

5.5.6 Technical reproducibility of Nm mRNA count of pharyngeal carriage samples

The reproducibility of the NanoString nCounter platform for technical variability was assessed using Nm positive pharyngeal samples, which had been gene counted in pervious different runs. The randomly selected samples showed high technical reproducibility between the replicate samples with a correlation of R^2=0.98 (Figure 5.8).

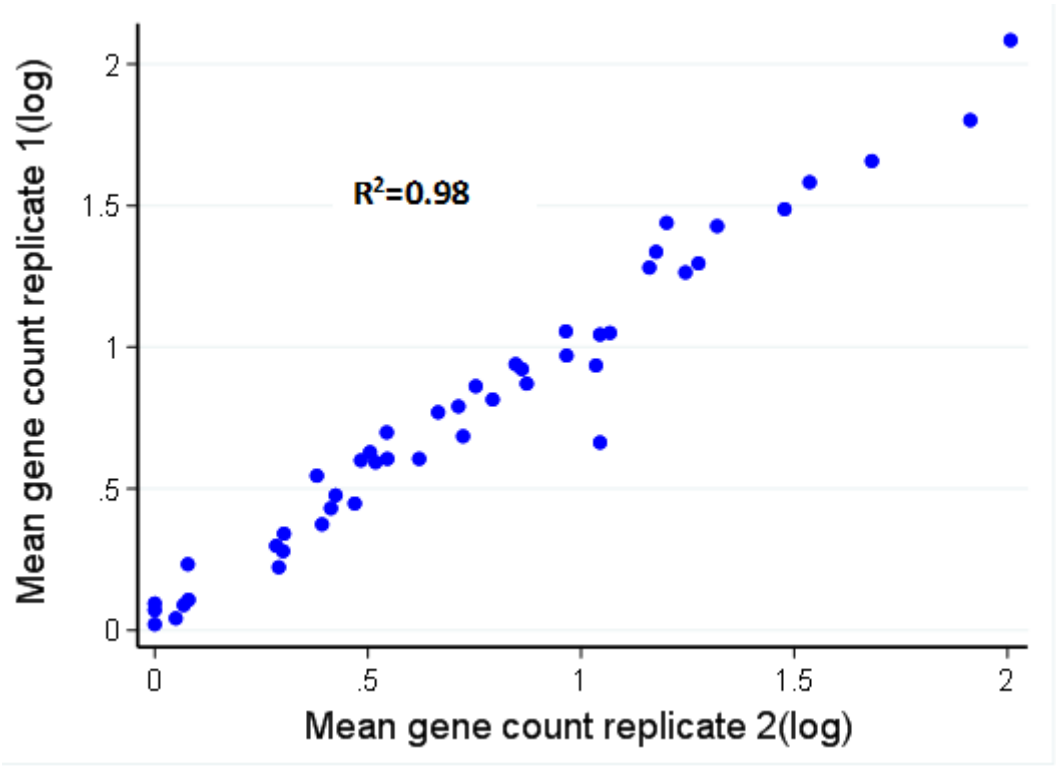


Figure 5.8 NanoString technical replicates showing the reproducibility of technical replicates of Neisseria meningitidis positive pharyngeal swab samples

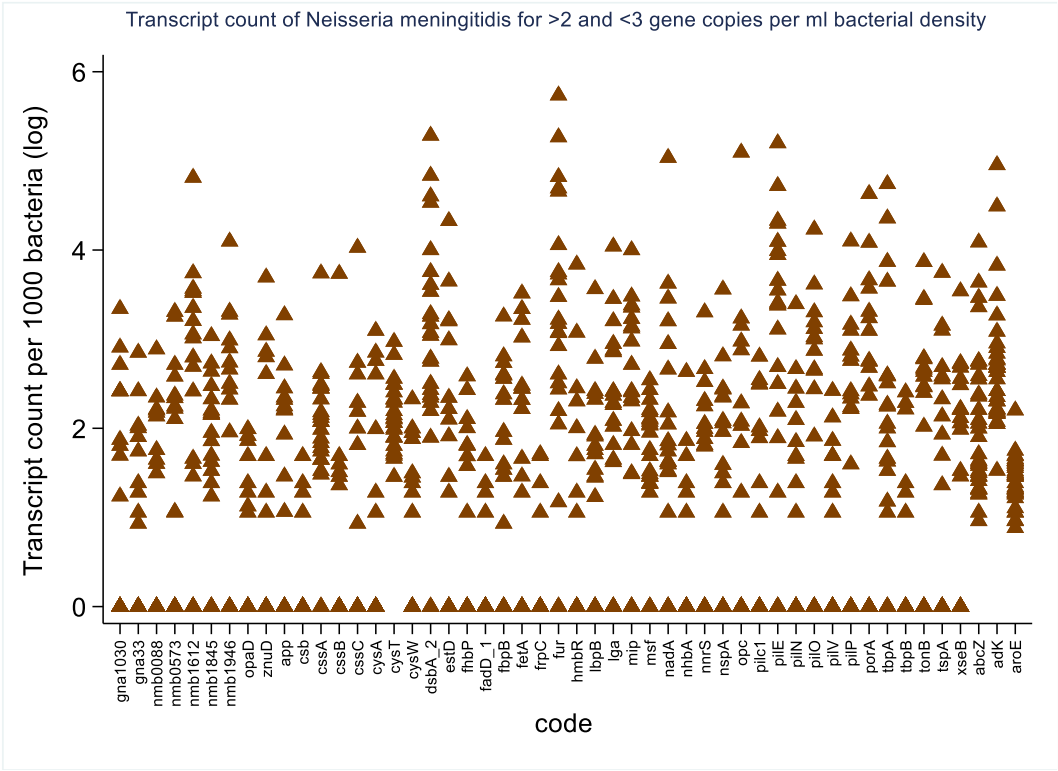
5.5.7 Gene expression profile by bacterial carriage density

To compare transcript count by carriage density, a scatter plot was done using the log transcript count per 1000 bacteria for two groups according to their bacterial carriage density.

The expression of genes from high and medium bacterial density is demonstrated in (Figure 5.9A and Figure 5.9B) respectively. Most genes in medium density carriers had transcript counts >log 1 (Figure 5.9A). In the high density group, there were two genes with negative log transcript counts (Figure 5.9B). Both high and medium density groups had genes that were switched off (that is with 0 transcript count values) regardless of density.

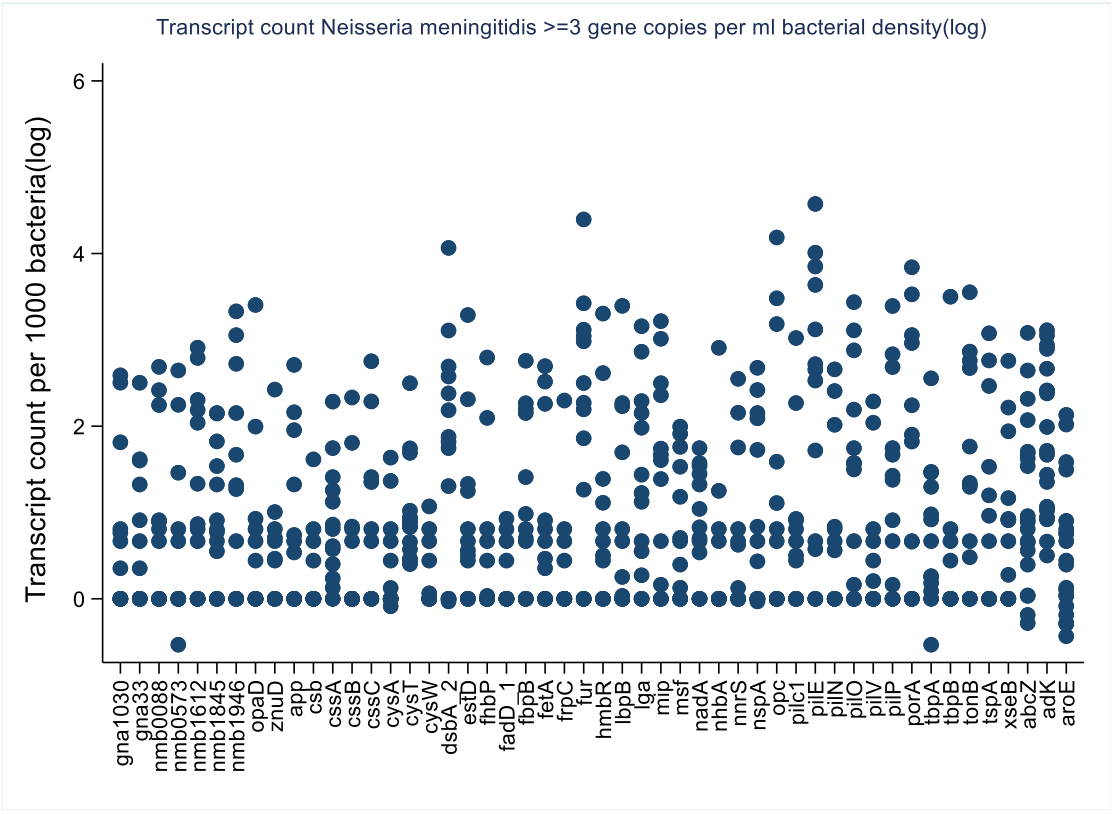
In general, high density samples had lower gene expression than low density samples. This is demonstrated clearly in (Figure 5.9C), which shows the overlay of the gene count from the two density groups.

A.


Transcript count of Neisseria meningitidis for >2 and <3 gene copies per ml bacterial density
Transcript count per 1000 bacteria (log)
0
2
4
6
gna1030
gna33
nmb0088
nmb0573
nmb1612
nmb1845
nmb1946
opaD
znuD
app
csb
cssA
cssB
cssC
cysA
cysT
cysW
dsbA_2
estD
fhbP
fadD_1
fbpB
fetA
frpC
fur
hmbR
lbpB
lga
mip
msf
nadA
nhbA
nnrS
nspA
opc
pilc1
pilE
pilN
pilO
pilV
pilP
porA
tbpA
tbpB
tonB
tspA
xseB
abcZ
adK
aroE
code

B.

C.


Transcript count Neisseria meningitidis >=3 gene copies per ml bacterial density(log)
Transcript count per 1000 bacteria(log)
0
2
4
6
gna1030
gna33
nmb0088
nmb0573
nmb1612
nmb1845
nmb1946
opaD
znuD
app
csb
cssA
cssB
cssC
cysA
cysT
cysW
dsbA_2
estD
fhbP
fadD_1
fbpB
fetA
frpC
fur
hmbR
lbpB
lga
mip
msf
nadA
nhbA
nnrS
nspA
opc
pilc1
pilE
pilN
pilO
pilV
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porA
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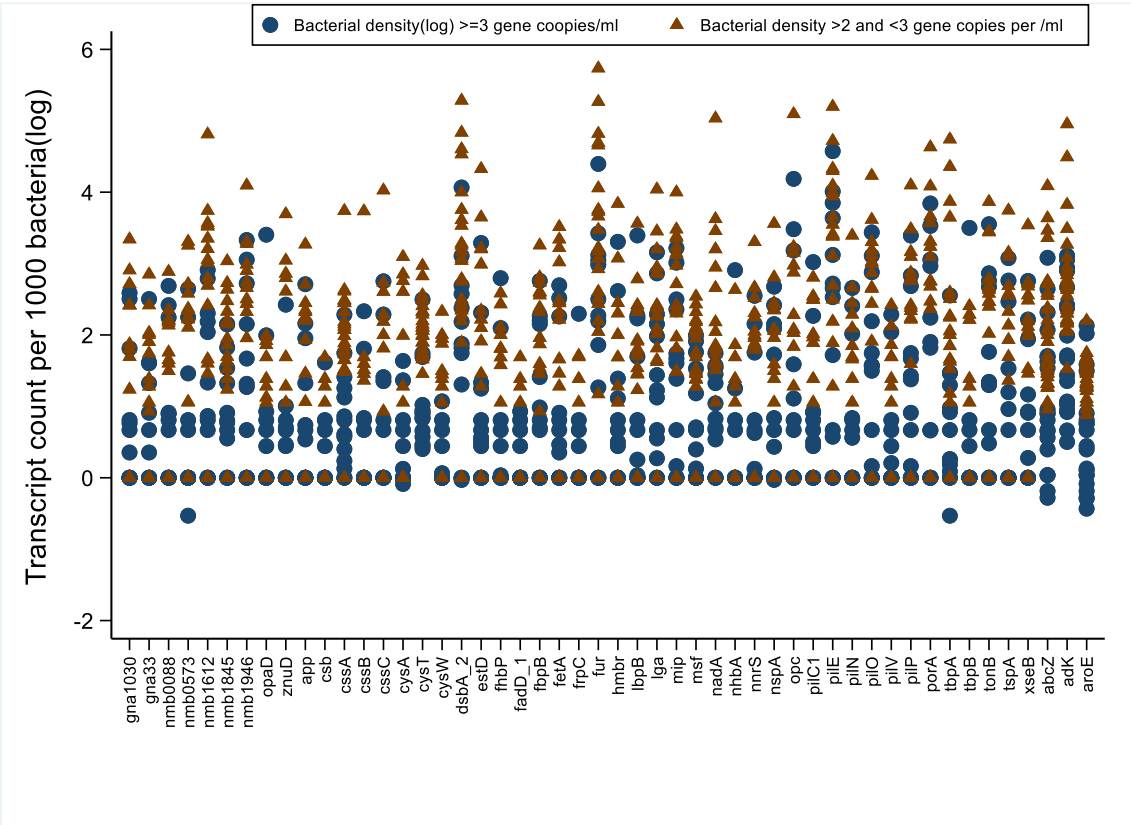
tbpB
tonB
tspA
xseB
abcZ
adK
aroE

D.

.



C.

Figure 5.9 Scatter graphs of Neisseria meningitidis gene expression profile (n=53) for 47 genes

Each dot is the log10 transcript count per 1000 bacteria for one subject. The y-axis shows log10 transcript counts per 1000 bacteria and the x-axis shows the gene names. Transcript counts with zero values were changed to one before log transformation for the sake of avoiding getting undefined results. 3A shows the gene expression from low density samples (gene copies <1000), 3B shows the count from high density samples (gene copies >1000),), and 3C shows the two groups overlaid. Blue circles indicate samples with bacterial density log value >= 3 and red triangles indicate those with bacterial density <3.

5.5.8 Data distribution of gene count and normality testing

In order to inform the statistical analysis, the distribution of the transcript count data for each gene was visually assessed on scatter plots. These are shown in Appendix C. Most of the expressed genes had a linear relationship with bacterial carriage

density. On the histograms that include the unexpressed counts (Figure 5.10A), all the genes had a positive skewed distribution and the data was transformed to its log 10 value before performing linear regression (Figure 5.10). The data for *porA* are shown as an example (Figure 5.10A, Figure 5.10B). The inverse normality plots for the log10 transformed data of each gene was linear, indicating a normal distribution (Figure 5.11).

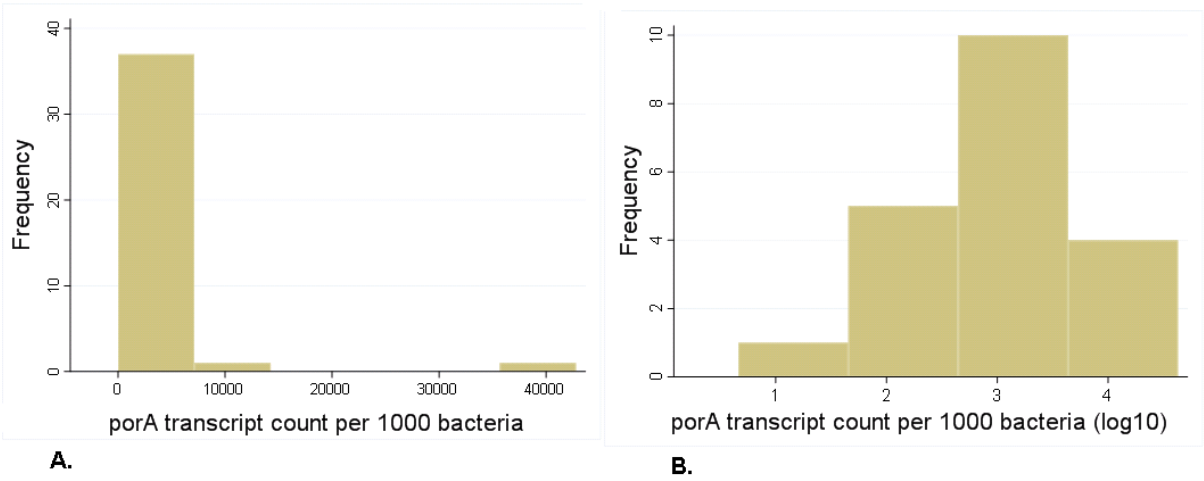


Figure 5.10 Frequency distribution of porA transcript count per 1000 bacteria A. untransformed data B. data transformed to log 10

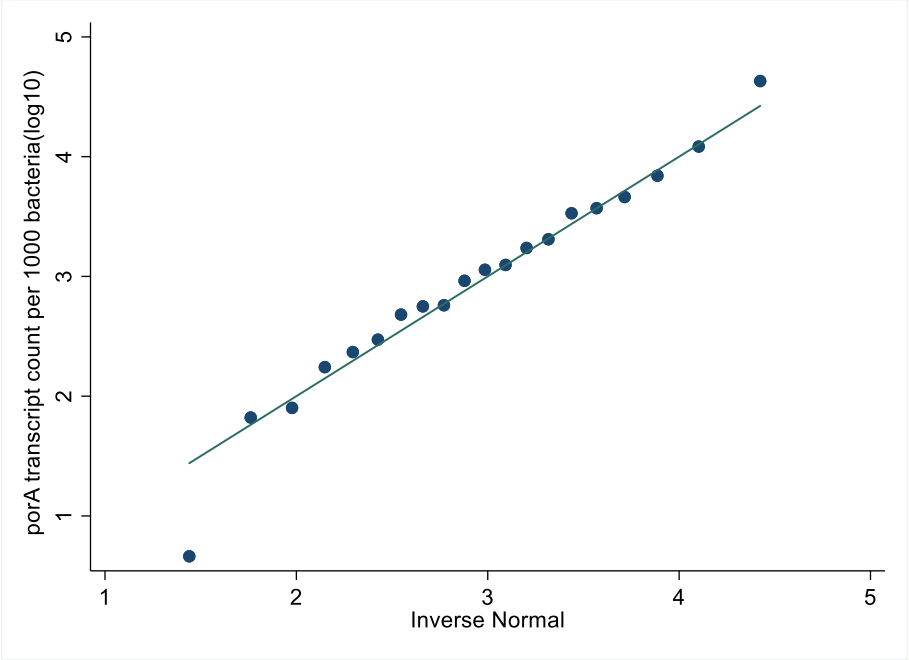


Figure 5.11 Inverse normal plot of the transcript count per 1000 bacteria (log10) of porA gene

5.5.9 mRNA expression and bacterial carriage density

To assess the relationship between carriage density and gene expression, regression analyses were performed for 42 of the 47 genes that had >10 independent observations. The genes with too few positive observations to permit linear regression were c*sb, tbpB, frpC, nhbA* and *fadD1.* All 42 genes analysed had a negative association with bacterial carriage density (decreasing gene expression with increasing density) in the unadjusted analysis (Table 5.3). Six genes (*cssA, cysA, cysT, cysW, msf, tbpA)* had a strong negative association with bacterial carriage density ($p<0.001$) (Table 5.3). The negative associations between density and gene expression remained unchanged after normalising the transcript counts against the housekeeping genes.

5.5.10 The association of gene expression with bacterial carriage density using Spearman rank correlation

Using an alternative statistical method, the association was also assessed by Spearman rank correlation analysis. All 47 genes showed a negative association between gene expression and bacterial carriage density. There was strong negative association in five of the genes after performing Bonferroni correction for 47 genes. These genes were *cssA* (rs(34)=-0.724, *cysA* (rs(13) =0.797), *cysT* (rs(39) =-0.812), *nadA* (rs(26) =-0.640), *tbpA* (rs(29) =-0.781) (Table 02) (p=0.001 for all five).

Similarly, a negative association between gene expression and bacterial carriage density using Spearman rank correlation analysis was observed in the other two studies (SPIT and Portugal) (Table 5.4).

5.5.11 Trends of density and gene expression in paired longitudinal samples

Trends in bacterial carriage density and variation in gene expression in the same individuals over time were assessed. Density varied between visits (Figure 5.12). No specific trend in gene expression was observed, the example below showing the trend for one gene (*gna1030*) (Figure 5.13).

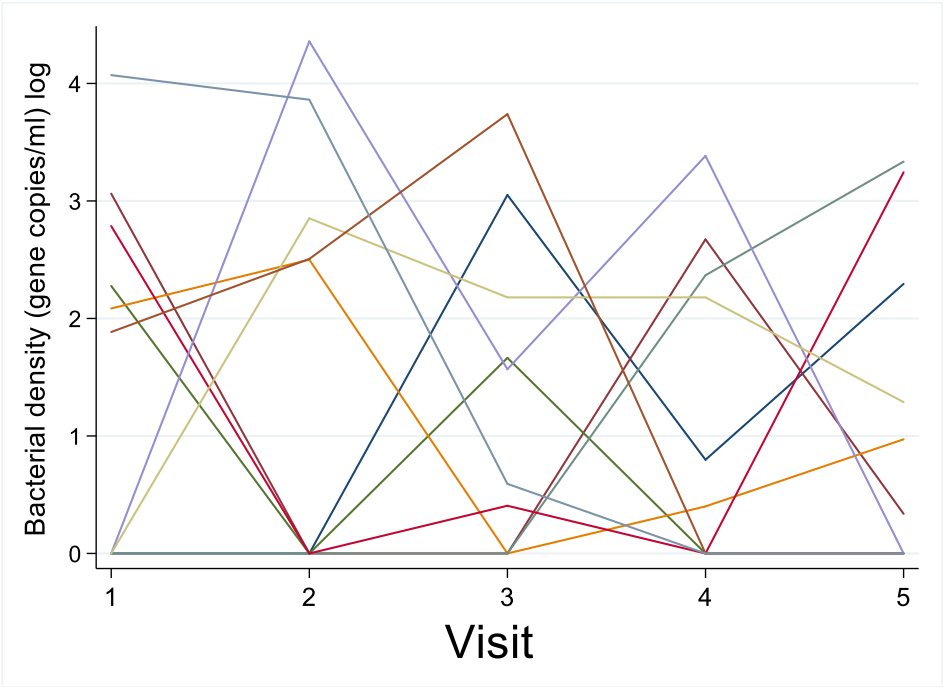


Figure 5.12 Bacterial carriage density of Neisseria meningitidis from paired longitudinal BrisMenNHC samples, each line indicates an individual

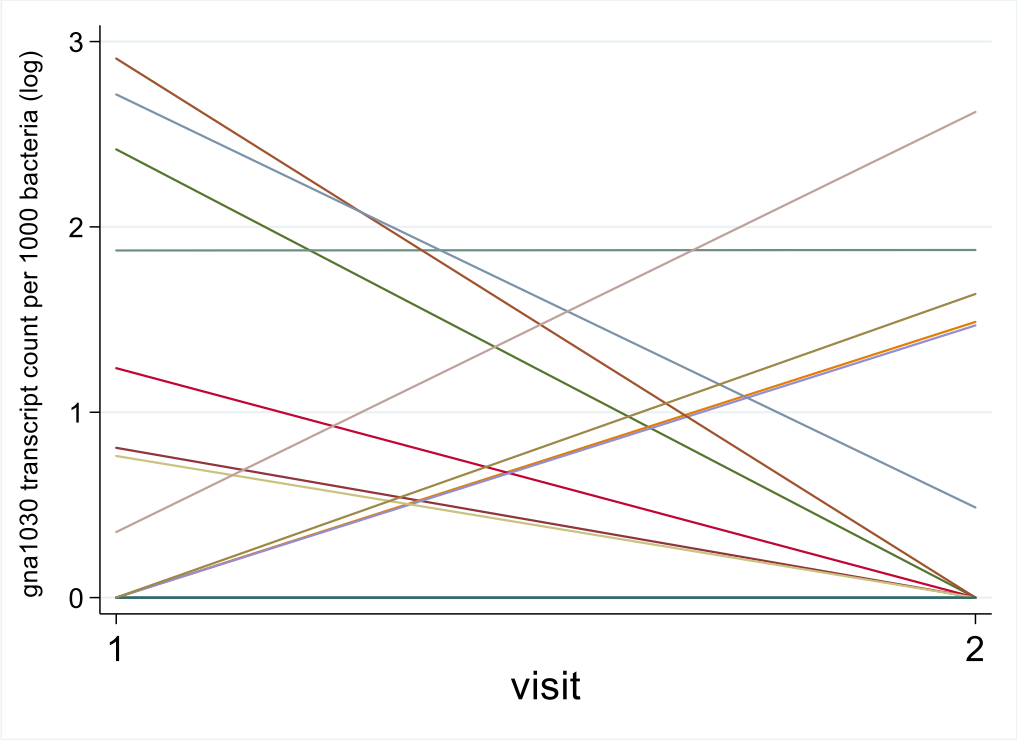


Figure 5.13 Trends of gna1030 expression between 12 paired longitudinal samples

5.5.12 Validation of NanoString transcript count results with RT-qPCR

As one validation of the NanoString results, *fhbP* transcript counts were compared with results from RT-qPCR. Out of the 48 samples tested using the nCounter platform,15 (31%) samples had >1 transcript count indicating gene expression and 33 (69%) had a zero-transcript count suggesting no expression. Of those 15 samples that had expression with NanoString, 6 samples gave the same result when tested with RT-qPCR and 9/15 samples were negative using RT-qPCR. From those with no expression using NanoString 4/33 samples were found to be positive using RT-qPCR (Table 5.2).

The *fhbP* gene expression result was grouped into two categories as expressed and not expressed. The inter-rater agreement between the two platforms was assessed using a Cohen's Kappa test. Overall, there was a 72% concordance between the two platforms.

Table 5.2 The number of samples which expressed fhbP with NanoString and RT-qPCR

NanoString SPRINT	Reverse transcriptase quantitative PCR		Total
	Not expressed	Expressed	
Not expressed	29	4	33
Expressed	9	6	15
Total	38	10	48

5.5.13 fHbp variants of BrisMenNHC samples tested with RT-qPCR

In order to compare RT-qPCR with WGS (see 6.5.8.), the expression of 48 BrisMenNHC carriage samples was analysed using RT-qPCR for the different variants of fHbp. From the 48 samples assayed, ten samples had fhbP gene expression. Of these, six were fHbP variant 1/family B, three were fHbP variant 2&3/family A and one had both variants.

Table 5.3 The association of gene expression and bacterial carriage density

Gene name	No of observation	coefficient	95% CI	*p value*
gna1030	16	-0.512	-1.164 0.140	0.114
gna33	16	-0.302	-0.975 0 .372	0.353
nmb0088	16	-0.127	-0.732 0.478	0.660
nmb0573	16	-0.793	-1.587 0.002	0.050
nmb1612	23	-0.773	-1.338 -0.207	0.010
nmb1845	25	-0.564	-0.916 -0.211	0.003
nmb1946	18	-0.420	-0.987 0.147	0.136
opaD	13	0.188	-0.774 1.149	0.676
znuD	14	-1.039	-0.916 -0.211	0.027
app	17	-0.483	-1.033 0.067	0.081
csb	7	ND	ND	ND
cssA(synx)	33	-0.954	-1.245 -0.662	**0.001**
cssB(siaB)	10	-0.217	-1.345 0.911	0.669
cssC(siaC)	13	-0.283	-1.308 0.742	0.556
cysA	13	-1.240	-1.851 -0.629	**0.001**
cysT	38	-0.921	-1.087 -0.755	**0.001**
cysW	13	-0.837	-1.243 -0.430	**0.001**
dsbA_2	32	-0.759	-1.280 -0.238	0.006
estD	19	-1.001	-1.631 -0.369	0.004
fhbP	14	-0.376	-1.127 0.376	0.298
fadd1	7	ND	ND	ND
fbpB	22	-0.414	-0.922 0.094	0.105
feta	19	-0.696	-1.362 -0.030	0.042
frpC	7	ND	ND	ND
fur	30	-0.606	-1.217 0.005	0.052
hmbR	15	-0.591	-1.388 0.207	0.134
lbpB	21	-0.463	-1.079 0.152	0.132
iga	26	-0.475	-0.955 0.005	0.052
mip	26	-0.480	-1.003 0.044	0.071
msf	28	-0.649	-0.937 -0.360	**0.001**
nadA(gna1994)	26	-0.865	-1.403 -0.327	0.003
nhbA(gna2132)	9	ND	ND	ND
nnrS	17	-0.616	-1.172 -0.058	0.033
nspA	21	-0.583	-1.087 -0.078	0.026
opc	16	-0.176	-1.311 0.959	0.744
pilC1	16	-0.550	-1.208 0.109	0.095
pilE	28	-0.343	-0.997 0.311	0.291
pilN	16	-0.341	-0.991 0.309	0.280
pilO	21	-0.619	-1.208 -0.029	0.041
pilV	13	-0.326	-0.991 0.339	0.304
pilP	22	-0.534	-1.123 0.054	0.073
porA	20	-0.549	-1.133 0.036	0.064
tbpA	29	-1.390	-0.959 0.278	0.259
tbpB	9	ND	ND	ND
tonB	22	-0.468	-0.994 0.058	0.078
tspA	17	-0.341	-0.959 0.278	0.259
xseB	22	-0.577	-1.041 -0.113	0.017

Table 5.4 Spearman rank correlation between bacterial carriage density and gene expression for BrisMenNHC, SPIT and Portugal study samples

BrisMenNHC				SPIT			Portugal		
Gene name	**Number of observations**	**Spearman's rho**	***P-value***	**Number of observations**	**Spearman's rho**	***P-value***	**Number of observations**	**Spearman's rho**	***P-value***
gna1030	16	-0.182	0.499	13	-0.588	0.035	11	-0.773	0.005
gna33	16	-0.259	0.333	6	-0.657	0.156	12	-0.846	0.001
nmb0088	16	-0.085	0.754	6	-0.657	0.156	8	0.000	1.000
nmb0573	16	-0.309	0.245	17	-0.779	0.001	12	-0.846	0.001
nmb1612	23	-0.515	0.012	12	-0.790	0.002	13	-0.742	0.004
nmb1845	25	-0.510	0.009	2	-1.000		20	-0.860	0.000
nmb1946	18	-0.166	0.510	13	-0.489	0.090	14	-0.481	0.081
opaD	13	-0.115	0.707	2	1.000		7	-0.929	0.003
znuD	14	-0.618	0.019	2	-1.000		8	-0.929	0.001
app	17	-0.289	0.260	5	-0.700	0.188	9	-0.717	0.030
csb	7	-0.464	0.294	0			3	-0.500	0.667
cssA	34	-0.724	0.001	6	-0.371	0.469	21	-0.848	0.000
cssB	10	-0.067	0.855	3	-1.000		8	-0.548	0.160
cssC	13	-0.214	0.482	8	-0.571	0.139	11	-0.518	0.103
cysA	13	-0.797	0.001	3	1.000	0.001	8	-0.857	0.007
cysT	39	-0.812	0.001	0			22	-0.898	0.001
cysW	13	-0.764	0.002	7	-0.714	0.071	12	-0.706	0.010
dsbA_2	33	-0.418	0.016	14	-0.688	0.007	20	-0.471	0.036
estD	19	-0.635	0.004	16	-0.779	0.001	11	-0.846	0.001
fhbP	14	-0.077	0.794	6	-0.600	0.208	9	-0.800	0.010
fedD_1	7	-0.893	0.007	0			11	-0.900	0.001
fbpB	22	-0.307	0.165	7	-0.750	0.052	14	-0.864	0.001
fetA	19	-0.426	0.069	12	-0.413	0.183	13	-0.692	0.009

BrisMenNHC				SPIT			Portugal		
Gene name	**Number of observations**	**Spearman's rho**	***P-value***	**Number of observations**	**Spearman's rho**	***P-value***	**Number of observations**	**Spearman's rho**	***P-value***
frpC	7	-0.143	0.760	5	-0.600	0.285	13	-0.758	0.003
fur	31	-0.340	0.061	14	-0.547	0.043	16	-0.350	0.184
hmbR	15	-0.443	0.098	8	-0.452	0.260	7	-0.536	0.215
lbpB	21	-0.142	0.541	17	-0.681	0.003	15	-0.836	0.001
iga	26	-0.190	0.351	13	-0.390	0.188	17	-0.596	0.012
mip	26	-0.256	0.207	15	-0.396	0.144	18	-0.416	0.086
msf	28	-0.550	0.003	14	-0.710	0.005	17	-0.721	0.001
nadA	26	-0.640	0.001	2	-1.000		16	-0.665	0.005
nabA	9	0.017	0.966	3	-0.500	0.667	2	-1.000	
nnrS	17	-0.331	0.195	5	-0.900	0.037	9	-0.600	0.088
nspA	21	-0.358	0.111	11	-0.546	0.083	16	-0.635	0.008
opc	16	-0.085	0.754	10	-0.188	0.603	13	-0.610	0.027
pilC	16	-0.406	0.119	4	-0.200	0.800	14	-0.939	0.001
pilE	28	-0.138	0.484	13	-0.374	0.209	18	-0.331	0.179
pilN	16	-0.232	0.387	7	-0.821	0.023	10	-0.952	0.001
pilO	21	-0.338	0.134	13	-0.308	0.331	14	-0.464	0.095
pilV	13	-0.192	0.529	4	-1.000		8	-0.762	0.028
pilP	22	-0.298	0.179	12	-0.350	0.265	14	-0.534	0.049
porA	20	-0.301	0.198	17	-0.380	0.133	15	-0.429	0.111
tbpA	29	-0.781	0.001	10	-0.212	0.556	19	-0.619	0.005
tbpB	9	-0.350	0.356	7	-0.821	0.023	5	-0.900	0.037
tonB	22	-0.147	0.417	10	-0.612	0.060	15	-0.561	0.030
tspA	17	-0.147	0.573	10	-0.697	0.025	10	-0.576	0.082
xseB	22	-0.326	0.139	9	-0.833	0.005	17	-0.632	0.007

5.5.14 Pattern of gene expression from BrisMenNHC, SPIT and Portugal carriage studies

To investigate consistency of gene expression from different carriage studies, gene expression of Nm in pharyngeal isolates from three studies was compared. All three showed a similar pattern of expression for all the genes tested (Figure 5.14). There was an 86% agreement between the two Bristol carriage studies (BrisMenNHC and SPIT) (k= 0.576, p<0.0001). There was a similar level of agreement between BrisMenNHC and Portuguese study with 83.4% agreement (k=0.5, p<0.0001).

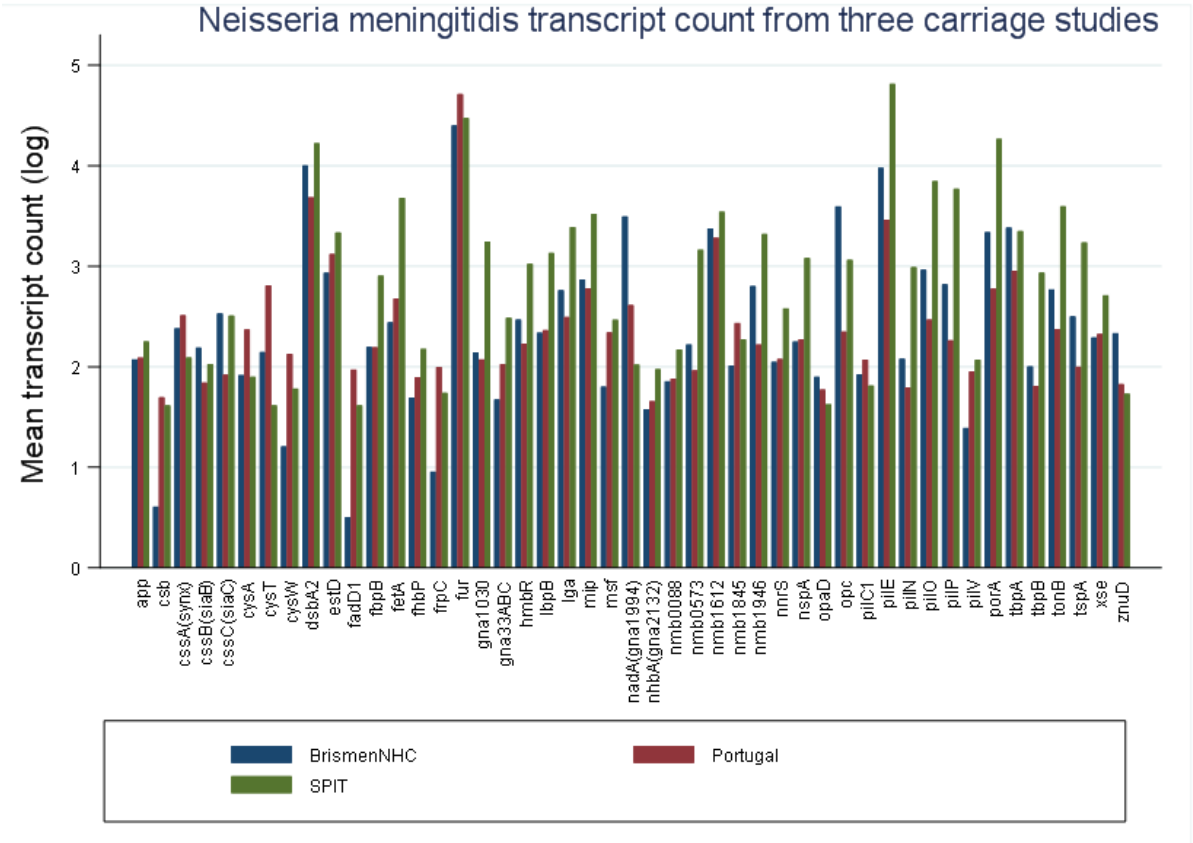


Figure 5.14 Neisseria meningitidis mean transcript count (log) from three carriage studies (blue is BrisMenNHC, red is Portugal, and green is SPIT carriage studies).

Table 5.5 Results of interrater reliability of transcript count of Neisseria meningitidis between three studies

Studies Pairwise Comparison	**Agreement**	**Expected agreement**	**Kappa**	**Standard error**	**Z**	**prob>Z**
BrisMenNHC with SPIT study	86%	64.0%	0.576	0.0923	6.24	0.0001
BrisMenNHC with Portuguese study	83.4%	66.0%	0.511	0.0923	5.53	0.0001
Portuguese with SPIT study	80.8%	66.0%	0.435	0.0923	4.71	0.0001

5.6 Discussion

This is the first study to directly detect and count Nm genes from pharyngeal carriage samples. Forty-seven different meningococcal genes were successfully profiled from pharyngeal carriage samples in three different studies. This study advances our knowledge of the genes involved in colonisation and carriage, and the expression of different genes of meningococcus in their natural environment. The variation of gene expression patterns was also examined, and the correlation was measured between gene expression and density of carriage.

Use of the NanoString nCounter system will improve with experience. This technology has the power to overcome some of the usual difficulties with studies of gene expression, such as the ability to obtain unbiased absolute gene counts for multiple genes at a time (151, 152). Limitations include the inability to prepare a specific probe for some Nm genes because of the close relatedness of the Nm genome with that of other neisseria species.

Fur, dsbA_2, pilE, opc, opa, and *nadA* were the main genes with a relatively high expression in the pharynx of carriers, suggesting a major role in colonisation. It is well established that the survival and pathogenesis of Nm depends on the ability to acquire iron within the host (94). Previous studies have identified genes which respond significantly to iron modulation in a positive or negative way (94, 141, 153). *Fur*, the most highly expressed gene is a well-known regulator of gene transcription of iron responsive genes(154, 155). High expression of *fur* was seen in all the three sample sets analysed (BrisMenNHC, SPIT and Portuguese carriage studies). This shows the importance of this gene for the survival of the organism when encountering a low iron environment during infection of the human host (141, 156). This gene regulates iron

balance between obtaining enough iron to grow and avoiding excess iron which can be toxic for the bacterial cell. This gene is multi-functional and is known to be important for energy metabolism, oxidative stress, acid tolerance, essential in small regulatory RNA metabolism (154, 157) and others.

Another highly expressed gene was *dsbA_2,* a periplasmic thiol disulphide oxidoreductase protein folding catalyst important in the folding of periplasmic and membrane proteins in Nm (158).

A third highly expressed gene in most individuals was *pilE,* a major subunit of type IV pili which helps the bacteria to adhere to the host cell (159). *pilE* is highly variable and key in pathogenesis and virulence of Nm. *pilE* is another multifunctional gene, important for attachment of the bacteria to the host cell, twitching motility, call aggregation and biofilm formation, adhesion to the host cell and intracellular signalling (159-161). Expression of *pilE* is important in the attachment of the bacteria to the host epithelial and endothelial cells whereas non-piliated bacteria cannot adhere to the surface of the cell(162). It is also important in keeping the cellular integrity of the bacteria. Type IV pili and *opa* are the major adhesion proteins that facilitate anchoring of Nm to the host cell which is a critical process for the attachment and survival of the bacteria(163). It was reported that in vitro studies of carriage isolates lose their type iv pili(164), This is a surprising finding given their function, and our study shows that carriage isolates taken from three studies express *pilE* relatively highly.

The association analysis showed that expression and bacterial carriage density were negatively associated, with five genes showing a strong negative association. A similar association was seen in the three different carriage studies. One explanation for this negative trend may be because the bacteria form micro-colonies at higher densities

and become partially dormant, or that some organisms may no longer be live. The density measurement is of bacterial DNA as opposed to live colonies. Dormancy or death of the organisms at higher bacterial density is supported by the persistent negative association after normalising for the stably expressed housekeeping genes.

Density of carriage and gene expression varied between paired longitudinal samples taken at 1-5-month intervals. It is probably not surprising that bacterial growth and activity vary over time in the changing environment of the pharyngeal mucosa.

Genes which encode for proteins in currently licensed MenB protein vaccines, *fhbP, nadA, porA* and *NhbA* were evaluated. *PorA* was the most highly expressed gene among the 4CMenB encoding genes, being expressed in 26/39 individuals in the BrisMenNHC study. *nadA* was expressed in more (29/39) individuals, but this could be due to the presence of other neisseria species due to cross-reactivity of the *nadA* probe. *nhbA* and *fhbP* were expressed in less than half of the carriers. Even though the expression of *fhbP* was relatively low in these carriage isolates*, fhbP* expression in disease isolates is high(145). Low expression of *fhbP* in carriage means that fHbp protein-based vaccines may not have an impact on carriage and transmission, hence herd protection may not be gained. This study showed a higher proportion of variant 1/subfamily B *fhbP* expression, unlike a study in France (145).

The concordance between RT-qPCR and NanoString was good with a kappa of 72 concordance, but there were some discrepant results. The samples tested with RT-qPCR were double digested with on column TURBO DNA digest which could explain why some genes positive with NanoString were negative with RT-qPCR. This could not be checked with other genes because no samples remained. This was one drawback of this study, not being able to test as many genes as I would have liked.

The four samples that were negative with NanoString and positive with RT-qPCR could be due to the low quantities of RNA in these samples.

This novel study shows that we can detect and quantify Nm genes using the NanoString nCounter system from pharyngeal carriers. There are limitations of the technique arising from cross-reactivity with other bacteria and low RNA quantity, and further validation is needed (also discussed in Chapters 6 and 7). Expression is important for a gene to be recognised by the immune system and may indicate potential for a protein vaccine to affect carriage and transmission. Highly expressed genes, especially if consistently present, could be used to propose new vaccine candidate proteins.

Chapter 6. Whole genome sequencing of BrisMenNHC carriage samples to confirm presence or absence of *Neisseria meningitidis* genes and predict level of *fhbP* expression

6.1 Introduction

A genome is a complete set of nucleotide bases representing a physical map of an organism's genetic content. The Neisseria genome contains 2,227,351 base pairs and 2158 predicted protein coding regions, of which 1158 have been assigned a biological role(23). The whole genome sequence gives us information about the presence or absence of genes in the genome. Studies have shown that some genes are not found in some strains of Nm such as *nadA* (165, 166).

fHbp is a surface-expressed lipoprotein that helps the bacteria to survive in the human blood by binding to human factor H thus downregulating the alternative complement activation pathway of the human immune system and assisting escape from killing (167, 168). fHbp is a component of two meningococcal protein vaccines, Bexsero and Trumenba. This protein is classified into three variants (Variant 1-3) and two sub-families (1-2) according to the amino acid sequence of the proteins.

The Variant 1, 2 and 3 classification is used by one vaccine manufacturer (Pfizer) and the sub- family A or B classification is used by another vaccine manufacturer (Novartis, now GSK). Variant 1 is equivalent to Subfamily B, Variant 2 and 3 make up Subfamily

A (Figure 6.1). For the rest of this chapter, the Variant 1, 2 and 3 classification will be used.

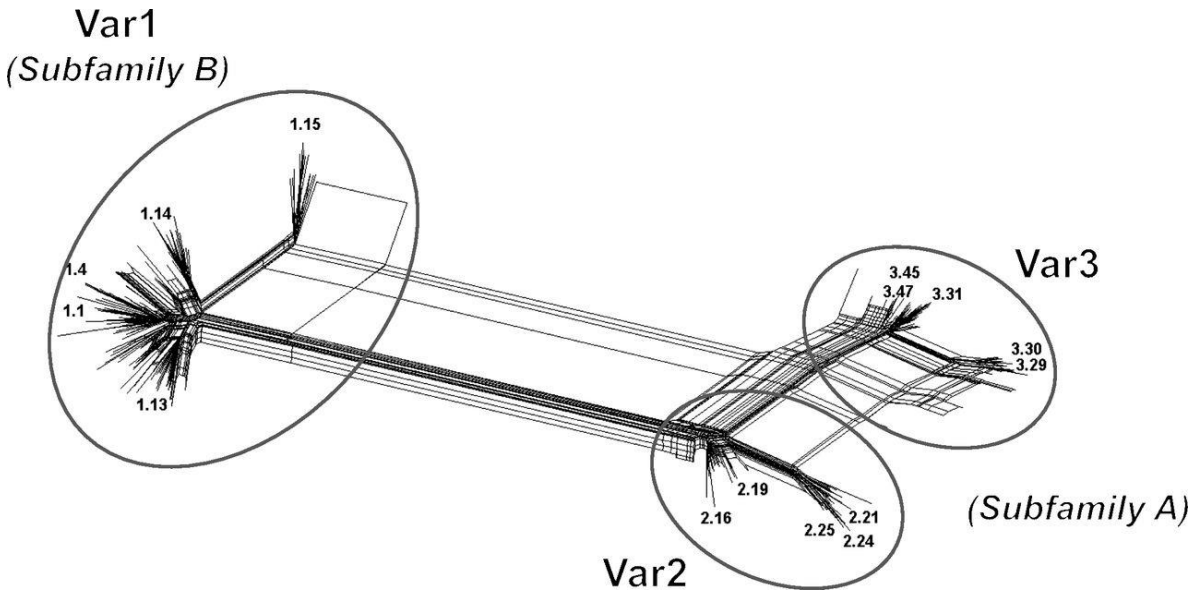


Figure 6.1 Phylogenetic distribution of fHbp by SplitsTree analysis showing the clustering of the proteins in the three main variants (Var1, Var2, and Var3) and in the two subfamilies (A and B)(169)

The level of *fhbP* expression in Nm isolates is associated with clonal complex, *fhbP* peptide and more strongly with IGR sequence (72). The IGR sequence is a region between the *fhbP* and *cbbA* genes, as indicated in Figure 6.2, that contains the core promoter and regulatory sequences of *fhbP*.

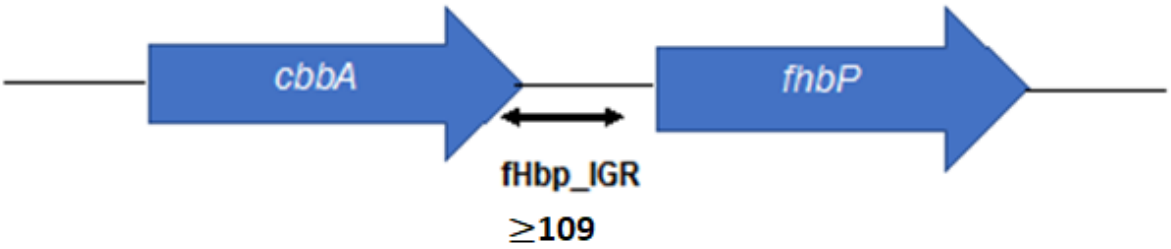


Figure 6.2 Schematic representation of the cbbA and fhbP genes and intergenic region (IGR) in the Neisseria meningitidis genome.

The blue arrows indicate the transcriptional orientation of the genes and the double arrow indicates the IGR containing the promoter signal of fhbP. (bP) base pairs

Clustered IGR sequences have been used successfully to predict the level of *fhbP* expression in disease isolates (72). Biagini et al also predicted *fhbP* expression using mass spectroscopy and showed an association between particular promoter sequence clades with *fhbP* expression (169).

In order to confirm the presence or absence of 47 genes detected using the NanoString nCounter platform in BrisMenNHC *Nm* carriage isolates and to determine whether detected levels of *fhbP* expression are consistent with predicted levels, the whole genome sequences of 47 isolates were obtained and analysed.

6.2 Aims

To assess the presence or absence of selected genes in the genome of Nm and assess the level of *fhbP* expression according to clonal complex, peptide and IGR.

6.3 Objectives

- To undertake WGS of Nm isolates from BrisMenNHC carriage samples with gene expression results
- To assess the presence or absence of 47 Nm genes measured by the NanoString nCounter system in the genome of these isolates
- To characterise the isolates by clonal complex, sequence type, capsular group and *fHbp* diversity
- To compare observed and predicted levels of *fhbP* expression by clonal complex, peptide and IGR
- To compare identification of *fHbP* variants by WGS with RT-qPCR results
- To assess genetic diversity in paired longitudinal carriage samples

6.4 Methods

6.4.1 Culture of selective and enriched media for Nm

Of 53 BrisMenNHC samples stored in STGG in which gene expression had been tested previously using NanoString SPRINT profiler, 47 were cultured on GC selective media containing four antibiotics to minimize the growth of other micro-organisms. Six samples could not be cultured. 100µl of each STGG sample was plated out onto GC media, and incubated in 5% CO_2 at 37ºC for 72 hours with observation at 24, 48 and 72 hours for identification of typical colonies of Nm. One typical colony of Nm was sub-cultured onto CBA agar for 16 hours in the same incubation conditions as above. The

colonies were examined visually for cross-contamination with other atypical colonies and checked by Gram stain and oxidase test.

6.4.2 Gram stain and oxidase test

Gram staining was done to assess the Gram reaction as positive/negative and the shape of bacteria. The Gram reaction is based on differential staining with crystal violet-iodine complex and a counterstain, safranin. Gram positive bacteria retain the primary colour crystal violet; Gram negative bacteria such as meningococci do not retain the primary crystal violate colour after decolourisation of the primary colour and iodine complex. Gram staining procedure is described in (3.2.11). Oxidase testing was done to assess the production of cytochrome C oxidase by Nm which shows a colour change to purple in the process of oxidising tetramethyl-p-phenylenediamine to indophenols (3.2.10).

6.4.3 Biochemical testing for identification of Nm (API_NH)

Gram negative, oxidase positive diplococci from 47 Nm positive samples were tested with API_NH for their biochemical properties according to the manufacturer's protocol (3.2.12). API_NH is a battery of biochemical tests done to identify Nm by their utilisation of different carbohydrates. The test includes fermentation of glucose, fructose, maltose, sucrose, and enzymatic reactions ornithine decarboxylase (ODC), urease, lipase, alkaline phosphatase, B-galactodisase, proline arylaminidase, gamma-glutamyl transferase, indole production and penicillinase detection.

6.4.4 Sending Nm isolates to Manchester PHE

The Nm isolates (250µl sample suspended in TSB + 10% glycerol) were sent in dry ice to the PHE Meningococcal Reference Unit (MRU) Manchester for WGS.

6.4.5 DNA extraction

Bacterial DNA extraction was done by PHE MRU following the manufacturers' instructions using a semi-automated platform MagMAX KingFisher. If samples were contaminated in the initial run, a manual kit called Wizard® Genomic DNA Purification Kit (Promega) was used for extraction.

6.4.6 Sequencing of the isolates

WGS was done by PHE Colindale using an Illumina Hiseq 2500 sequencing platform and a Nextera XT kit was used to prepare a library file using 1ng DNA. The read length was 2 x 100bp paired end (dual indexed). The genome sequences were sent to the University of Oxford (Keith Jolley) to enter into the pubMLST database in the Meningococcus Genome Library. Each sequence was assigned a pubMLST ID (from 61935 to 61982) (170).

6.4.7 Identification of presence or absence of genes

Forty-seven Nm genes were systematically screened from the whole genome sequence using the Neisseria pubMLST database presence/absence status of loci

genome comparator tool. The pubMLST ID was used to retrieve the genome sequences from the database and the specific loci of each of the 47 genes were selected to do the analysis. Genes not identified by automatic scanning using the presence/absence analysis tool were scanned individually. Individual scanning involved the extraction of the gene sequences using the specific pubMLST ID numbers to identify genes that had a partial or truncated sequence or were difficult to assemble.

The alignment was done using the pubMLST BLAST link. The pubMLST ID was submitted to the isolates field and the sequence of each genome downloaded from pubMLST was pasted and aligned with the whole genome of Nm in the database. The presence of the gene was then assessed using the alignment percentage identity and the number of mismatches.

6.4.8 Genome analysis for sequence type and clonal complex analysis

The genomes of 47 Nm isolates were automatically searched and scanned using the pubMLST ID of the isolates in pubMLST website for the presence of 47 genes. The Sequence type (ST) and clonal complex (CC) were assigned based on the seven MLST genes (*abcZ, adk, aroE, fumC, gdh, pgm, pdhC*). The strain designation of the isolates for the *porA* and *fetA* genes were determined.

6.4.9 IGR identification

Identification of the IGR region igr_up_NEIS0349 was done in the Meningococcus Genome Library using the pubMLST ID. The IGR region sequences were obtained and aligned on BioEdit sequence alignment software to identify the polymorphic sites. The strain designations of each of the isolates were assessed.

6.4.10 Identification of fHbP variants

The variants of fHbp were identified using the pubMLST Neisseria species database. The fHbP allele number for each sample was obtained using the isolate ID and choosing the *fhbP* loci (NEIS0349) from the database. The different peptides were searched using *fhbP* alleles and peptide menu, and the summary of each *fhbP* allele was done using the peptides summary for their allele ID.

6.4.11 Identification of Nm genogroups

The capsular groups of the samples were identified using the pubMLST automatic gene search. Meningococcal capsules play an essential role in the virulence of the organism (171). Genes involved in polysaccharide biosynthesis, termed cp (for capsule synthesis) are located on a single chromosome (20), and are given a region name (6 regions) from A-D, D', and E (Figure 6.3). Genes in region A encode enzymes for biosynthesis of the capsular polysaccharide, e.g. *csb, csc, csw*, and were used to identify the capsular groups of the isolates. Regions B and C are used in translocation of high molecular weight polysaccharides to the cell surface, Regions D and D' are involved in lipopolysaccharide synthesis (20). Meningococci which lack the capsule synthesis genes and contain the capsule null locus (cnl) are indicated.

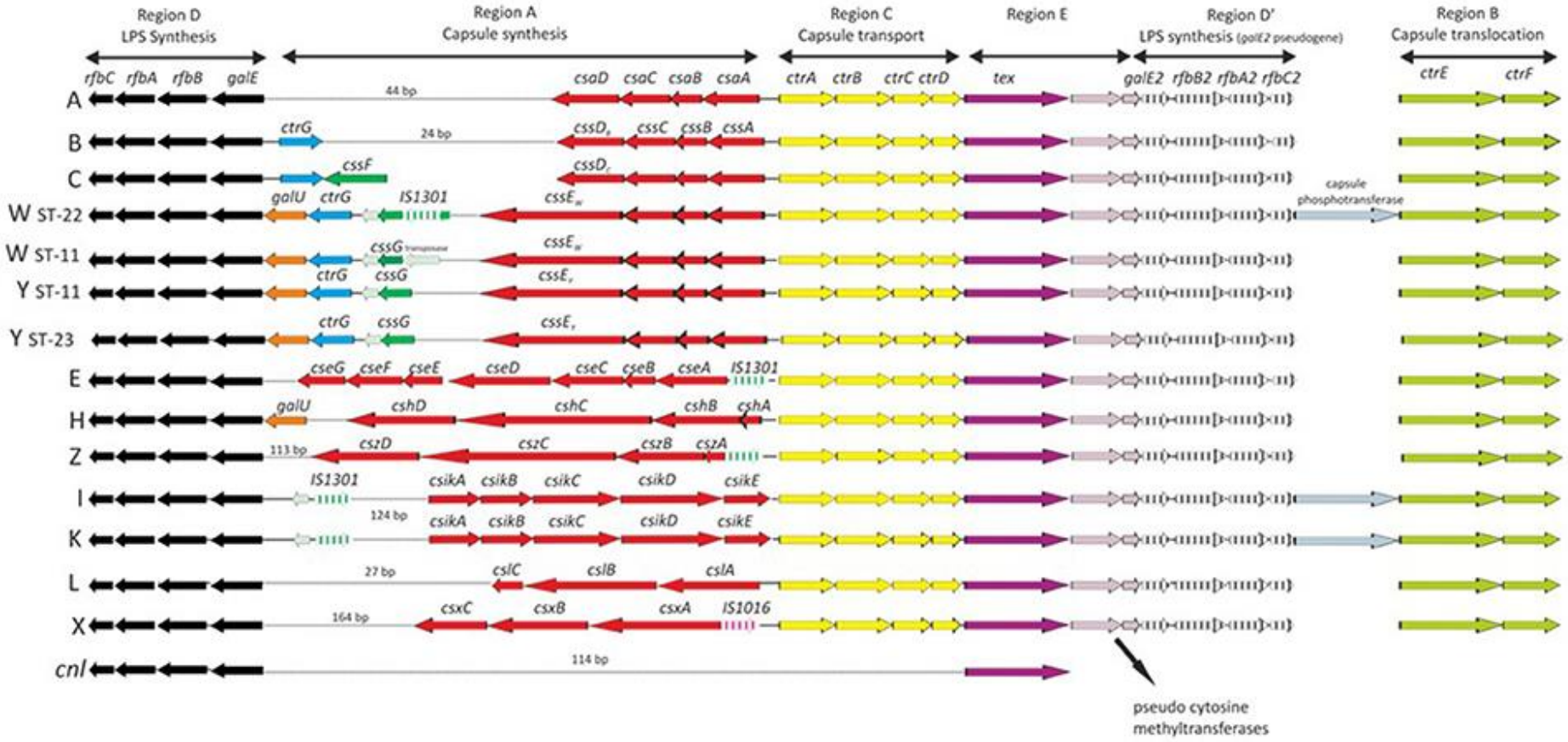


Figure 6.3 Genetic organisation of the capsule locus among Neisseria meningitidis serogroups (20)

6.4.12 Statistical analysis

Genomic analysis of the isolates was done in pubMLST using the genome comparator analysis tools with the annotated reference genome as a comparator. The output was exported to Stata 15 to plot some of the descriptive bar graphs. splitTree4 software was used to plot the phylogenetic tree using a distance metric after extracting the sequences of the genes from pubMLST.

6.5 Results

6.5.1 Nm growth on GC and CBA

To obtain Nm isolates for WGS, Nm positive swab samples that had gene expression results were cultured. Of 53 samples, 47 were successfully cultured. One sample yielded two distinct Nm colonies (different size and morphology) that were sent separately for sequencing. The two colonies had the same CC, ST, capsular group and strain designation, so were considered to be the same strain. This left 47 isolates for analysis.

6.5.2 Presence of 47 genes in the 47 whole genome sequenced samples

To compare results obtained by WGS and the nCounter system, presence or absence of the 47 genes was checked. Of the 47 genes tested, 42 (89.4%) were present in all the genomes of the carriage isolates tested. Of genes not found using the automatic search and scanned individually on pubMLST database, the sequences were found to

be either partially present, fully present or absent from the genome. Partially present gene sequences were either at the end of a contig or were truncated. The pilin genes (*pilE* and *pilC_1*), *fetA, humR, tspA, iga, fadD1* were found in all samples as full or partial sequences.

Five genes were absent from some or all isolates. One gene (*frpC*) was absent from all isolates. *nadA* was absent in 32/47 (68.1%) of the samples. The capsule synthesis genes (*cssA, cssB* and *cssC*) were absent in 23/47 (48.9%) of isolates (cnl (n=14), capsule not determined (ND) (n=5), genogroup B (n=2) and genogroup E (n=2)).

6.5.3 NanoString gene counts in samples with absent genes

To validate the comparison further, NanoString transcript counts were examined in samples where genes had not been found by WGS.

The *frpC* gene was absent from the genomes of all Nm isolates, yet eight samples had an expression result for this gene. Of the eight positives, seven had a gene count of <50 transcripts per 1000 bacteria and one isolate had 198 transcripts per 1000 bacteria.

Although the *nadA* gene was absent in 33 isolates, transcript counts for *nadA* were detected in 19 of these 33 isolates. The six highest *nadA* transcript counts (all >458-4222/1000 bacteria) were found in isolates that did not contain the nadA gene.

The expression of the cssA, cssB and cssC genes found in the capsule synthesis region A of Nm (Figure 6.3) were assessed in cnl isolates. Two of 14 cnl had no transcript count for *cssA*, 13/14 cnl samples had no expression for *cssB*, and *cssC*.

6.5.4 Genotypic diversity of BrisMenNHC carriage isolates

To document the genetic diversity of the isolates and inform subsequent validation (see 6.5.7.), their clonal complexes, genogroups and fHbp variants of the isolates were described. Thirteen different clonal complexes were identified (Table 6.1, Figure 6.4, Figure 6.5). Of the 47 samples, four (8%) were not assigned to any clonal complex (two genogroup B (ST-1167), two genogroup Y (ST-6798).

Table 6.1 Distribution of genogroups and fHbp variants of BrisMenNHC carriage isolates (N=47) within clonal complexes

Clonal complex	Frequency of isolates	Genogroup	*fHbp*_ variants
cc11	2	2 W	2 (var 1)
cc1157	8	3 E, 4ND, 1 B	8 (var 1)
cc162	1	1 B	1 (var 1)
cc198	7	7 cnl	6 (var 2), 1(var 1)
cc213	2	2B	2 (var 1)
cc22	3	3 W	3 (var 1)
cc23	5	5 Y	5 (var 2)
cc269	1	1 ND	1 (var 3)
cc32	2	1 cnl & 1 B	2 (var 1)
cc35	4	4 B	1 (var 1), 3 (var3)
cc41/44	1	1 B	1 (var 1)
cc461	1	1 B	1 (var 1)
cc53	6	6 cnl	6 (var 1)
cc not assigned	4	2 B, 2 Y	2 (var 1), 2 (var 2)

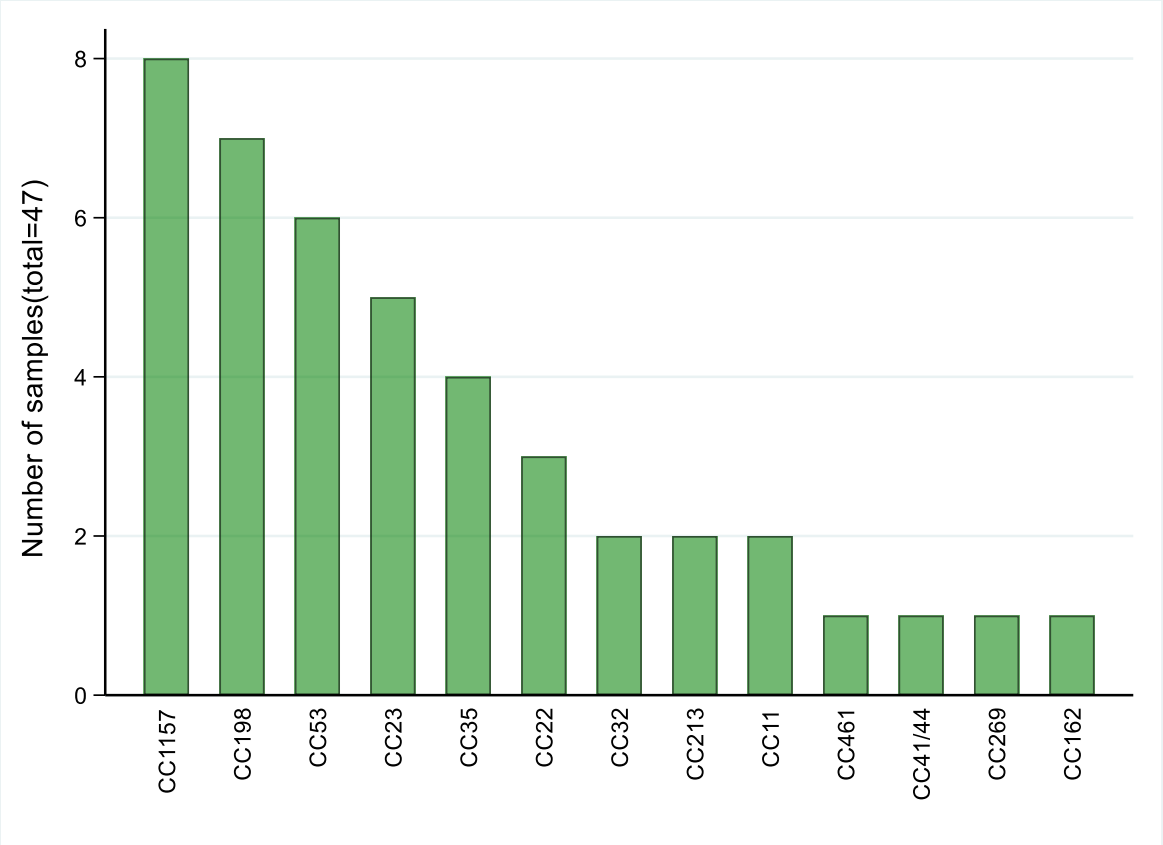


Figure 6.4 Distribution of the clonal complexes of BrisMenNHC carriage isolates

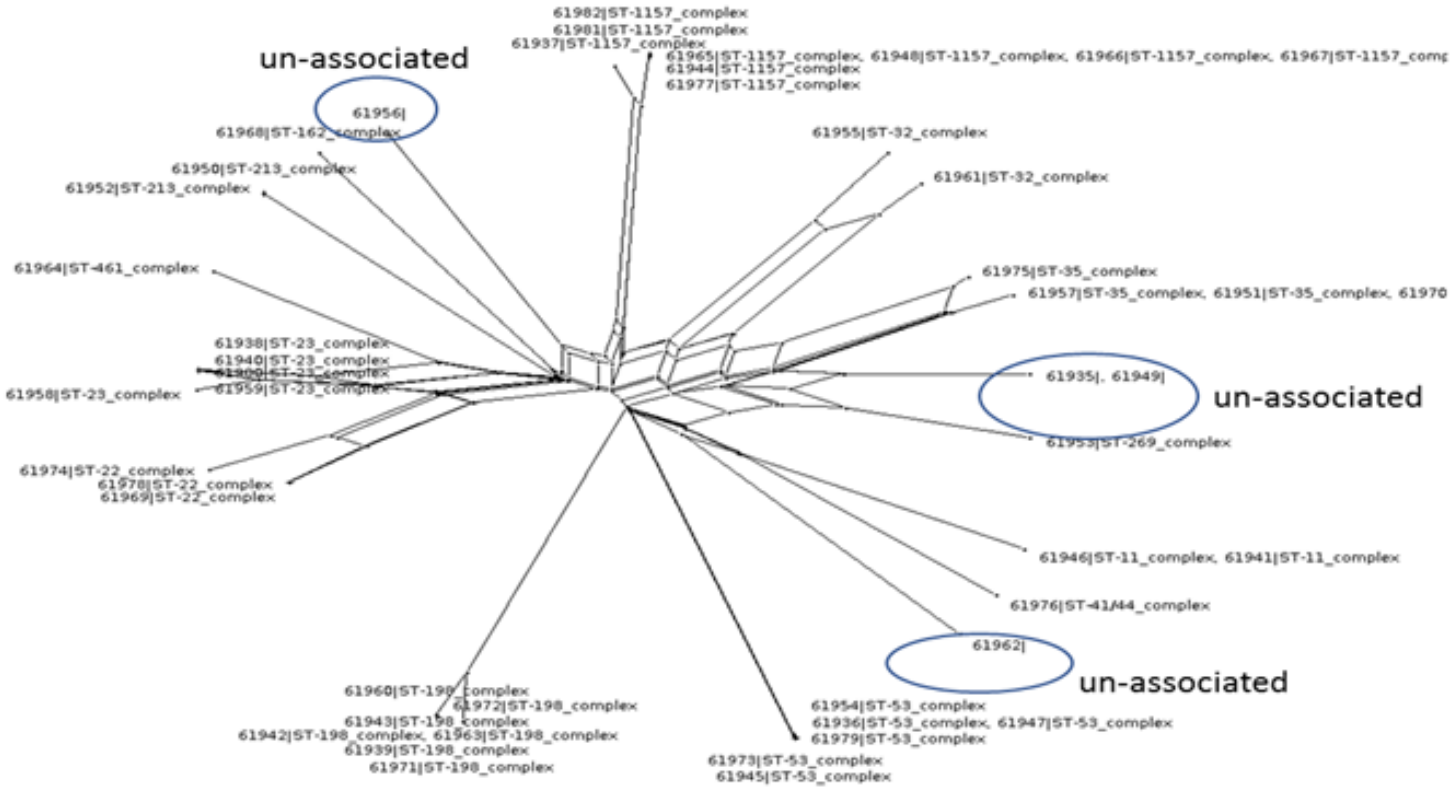


Figure 6.5 Analysis against all MLST loci showing Neisseria meningitidis clonal complex (ST complex) for 47 BrisMenNHC carriage isolates. The circles indicate the strains which are not associated with any clonal complex in pubMLST database

Of the genogroups, the highest number were cnl 14 (29.8%), 13 were genogroup B (27.7%), 7 (14.9%) genogroup Y, 5 (10.6%) genogroup W and 3 (6.4%) genogroup E (Figure 6.6, Table 6.1). In five (10.6%) samples, the genogroup could not be identified (ND) from the sequence. Genogroup B had the most diverse range of clonal complexes. Two genogroup W isolates belonged to the hypervirulent clonal complex cc11.

6.5.5 Distribution of Nm in different ST variants

The distribution of sequence types among the isolates was described The most frequent sequence type was ST-1157 found in 7 (14.9%) of the isolates (4 cnl, 3 E) (Figure 6.6)

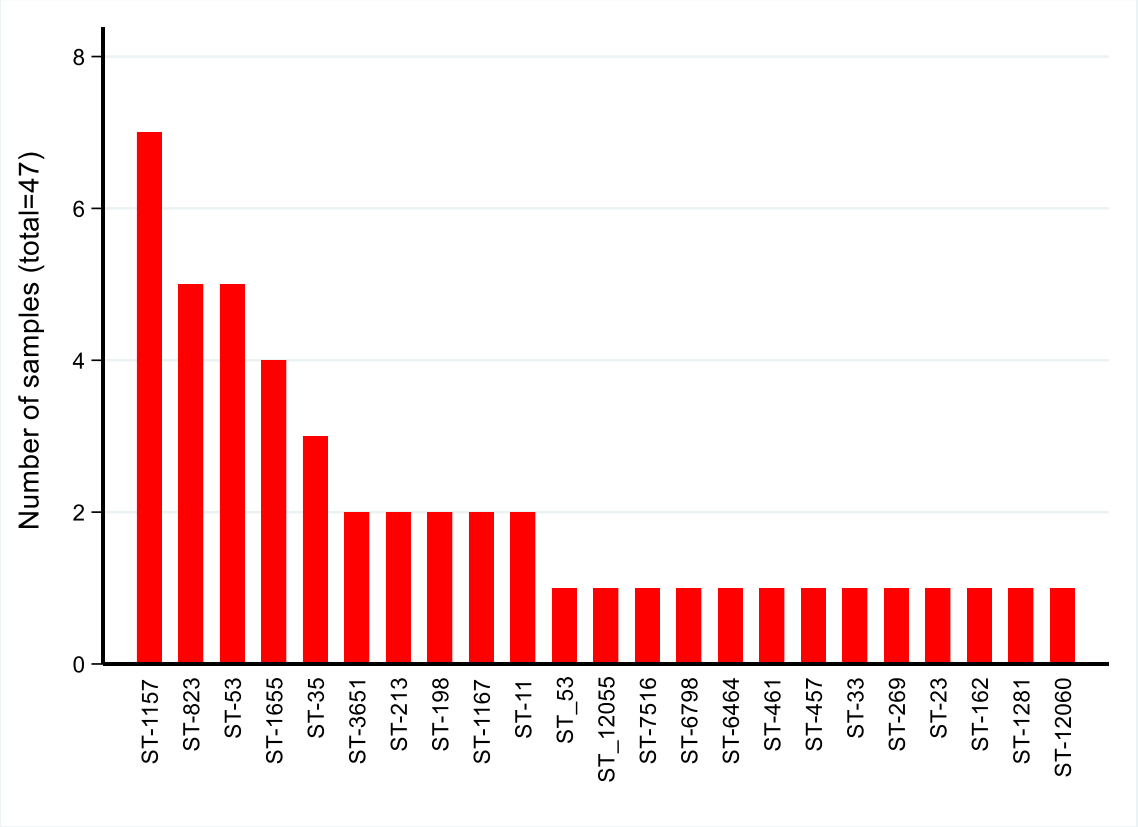


Figure 6.6 Distribution of sequence types of BrisMenNHC carriage isolates from the 47 Neisseria meningitidis positive isolates

6.5.6 Genetic diversity of fHbp in BrisMenNHC isolates

To also inform subsequent validation (see 6.5.7, 6.5.8), WGS analysis of *fHbp* variants and IGR sequences was described. Variant 1 (29/47, 61.7%) was the predominant variant in BrisMenNHC carriage isolates tested. Variant 2 was less frequent, 14 (29.7%) followed by Variant 3 accounting for four (8.5%) samples. The most frequent peptides were 209, 206, 2 and 26 (Figure 6.7).

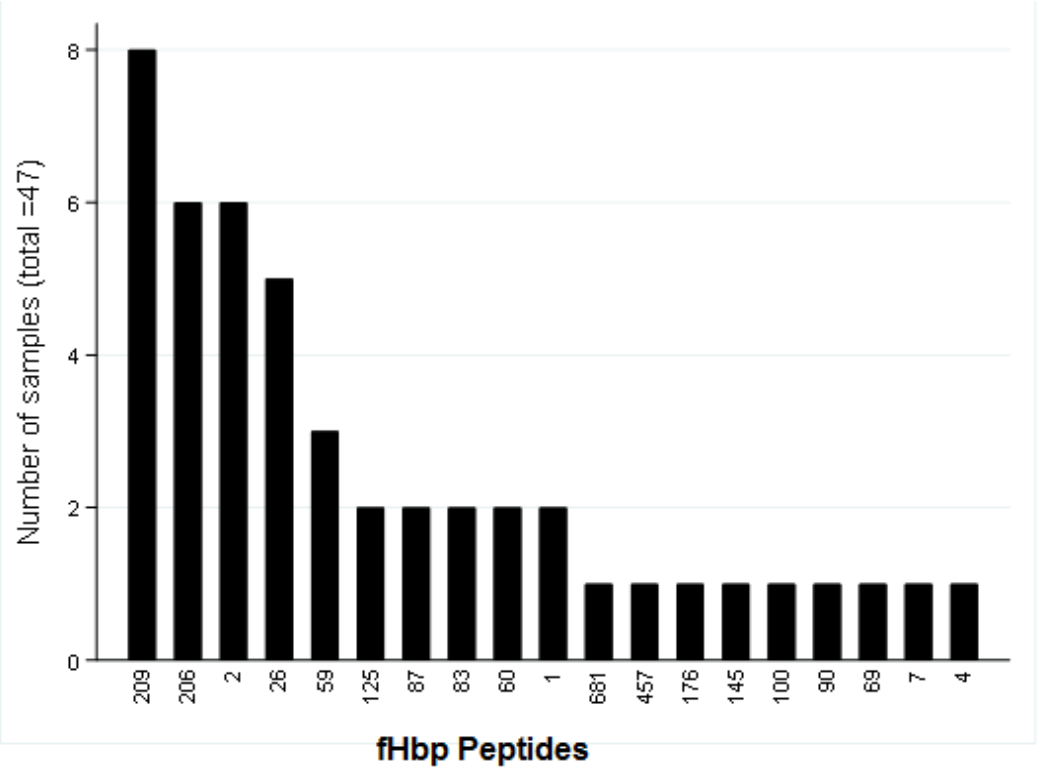


Figure 6.7 The distribution of fHbp peptides among 47 Neisseria meningitidis positive carriage isolates

Six *fHbp* IGR sequences were identified in 45 isolates, the commonest being *fhbP*_IGR 1 (Table 6.2). Two isolates did not have a recognised IGR. The alignment of fhbP_IGR sequences in the six alleles identified nine polymorphic sites (Table 6.2).

Table 6.2 The frequency of igr_up_NEIS0349 alleles in 47 BrisMenNHC carriage isolates

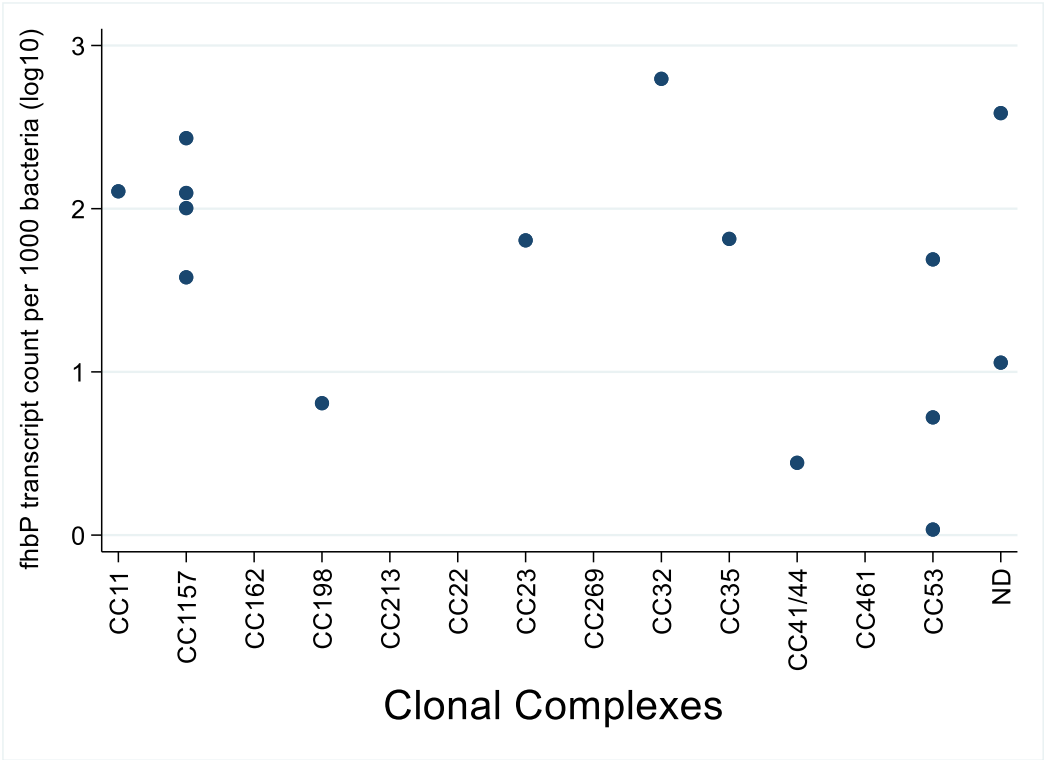
Igr_up_NEIS0349	Frequency	Percentage
1	24	51.1
3	9	19.2
8	8	17.0
No value	2	4.3
12	2	4.3
98	1	2.1
6	1	2.1

Figure 6.8 Intergenic region sequence alignment of fhbP showing the polymorphic sites between six different alleles of BrisMenNHC carriage isolates

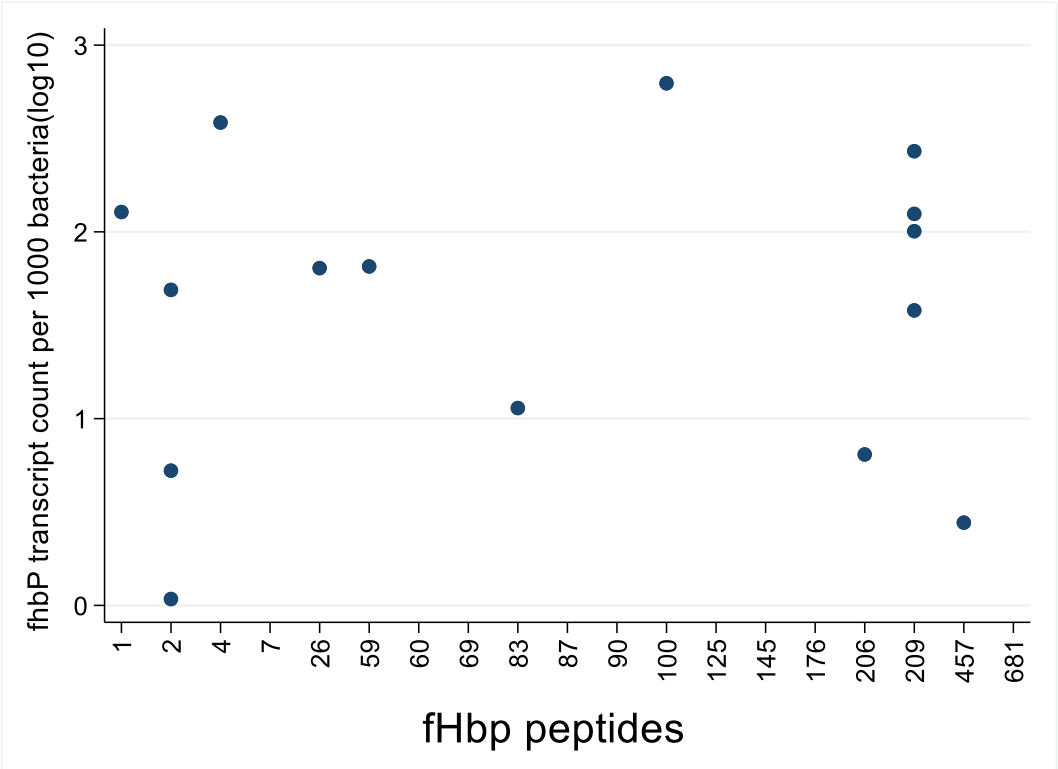
6.5.7 Expression of *fhbP* between different CC, fHbP peptides and IGRs and comparison with predicted levels

In order to validate the NanoString results, expression of the *fhbP* gene by clonal complex, peptide and IGR was compared with levels predicted from previous research. Expression levels of *fhbP* as measured by NanoString varied by clonal complex. Highest counts were seen in cc32 and cc1157 (n= 763/1000 and 316/1000 bacteria respectively, Figure 6.9A), some such as cc162, cc213, cc22, cc269 and cc461 showed no *fhbP* expression. Expression by *fhbP* peptide and IGR were also highly variable (Figure 6.9B and Figure 6.9C).

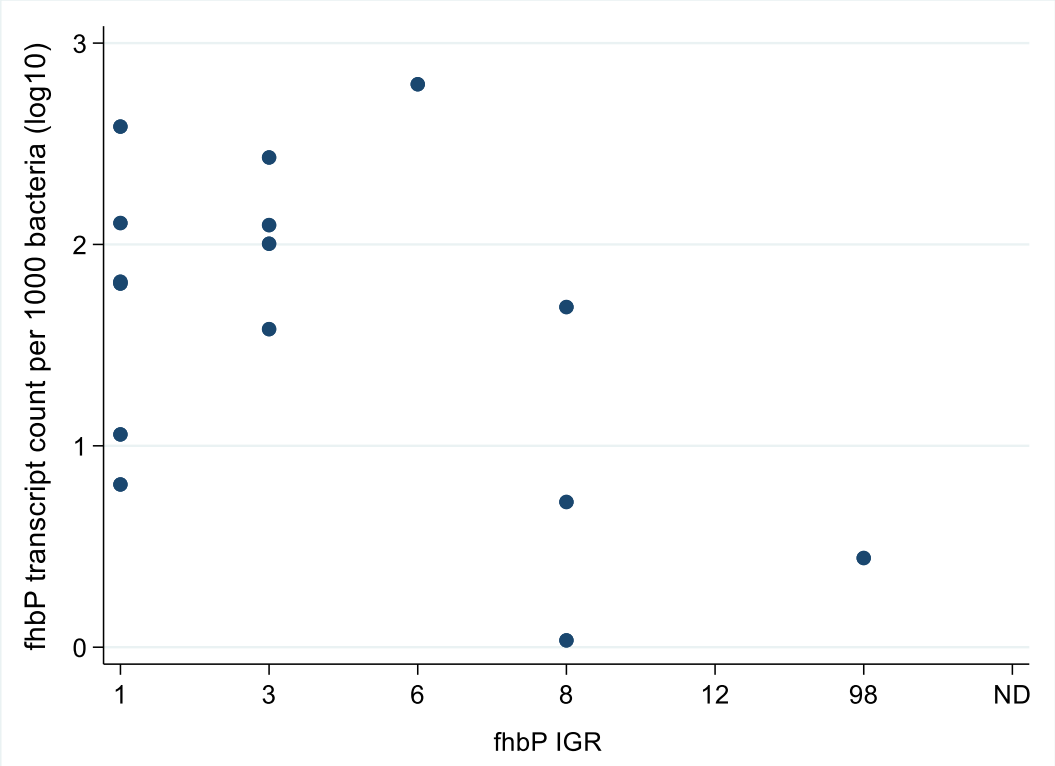
Of the clonal complexes (cc32, cc269) associated with higher *fhbP* expression (72), only one isolate in this study had *fhbP* gene expression. Similarly, of fHbp peptides (1 and 15) associated with higher *fhbP* expression, only one isolate had gene expression detected by NanoString. Therefore, numbers were too small to make a valid assessment. For IGR comparison, most samples were IGR_1, part of an IGR cluster predicted to have intermediate level of *fhbP* expression. The IGR_1 expression results showed a broad range of expression, and the one isolate in an IGR cluster predicted to have a high level of expression (IGR_6) had the highest gene expression of the samples tested.


fhbP transcript count per 1000 bacteria (log10)
0
1
2
3
CC11
CC1157
CC162
CC198
CC213
CC22
CC23
CC269
CC32
CC35
CC41/44
CC461
CC53
ND
Clonal Complexes

A.


fhbP transcript count per 1000 bacteria(log10)
0
1
2
3
1
2
4
7
26
59
60
69
83
87
90
100
125
145
176
206
209
457
681
fHbp peptides

B.



C.

Figure 6.9 Comparison of fhbP expression by A. clonal complexes B. peptides C. Intergenic region (IGR) alleles. Isolates with no fhbP expression by NanoString are not shown

6.5.8 Comparison of fhbP variant identification using RT-qPCR and WGS

As a validation of the RT-qPCR results in Chapter 5, fhbP variants confirmed by WGS were compared with those identified by RT-qPCR. Among the 10 samples tested with RT-qPCR for the three variants of *fhbP* (5.5.13), seven had the same *fhbP* variant when tested with WGS. Three samples had discordant results. Two isolates were Variant 2 by RT-qPCR but Variant 1 by WGS, and vice versa for the third isolate.

6.5.9 Genetic diversity between paired carriage samples in longitudinal study

To investigate genetic diversity in carriage samples taken from the same individual at different times, ten paired longitudinal samples were compared based on MLST allelic distance metrics and presented as a Neighbor-net network phylogenetic tree generated using the core MLST (Figure 6.10). From the 10 paired samples, four samples had a one-month interval, 3 a two-month interval, 1 a three-month interval and 2 a 5-month interval.

The ten paired samples belong to seven different clonal complexes. Most 9/10 of the paired samples were closely related between the pairs. One pair had different strains at the two visits (one of the 5-month interval pairs) with different genogroup, ST, *fhbP* variant and strain designation (ND: P1.22-1,14: F4-1: ST-35 (cc35) at visit two and ND: P1.5-1,10-26: F5-5: ST-6798 at Visit 6 (Table 6.3) and are denoted by alphabetical letters and numbers.

The ten paired samples were collected from 6 different schools. Of three pairs who carried the same ST (ST53) at all visits, (Table 6.3), two subjects were from the same school, both male, white ethnic and school year 13. There were no data on social links between them.

Table 6.3 The distribution of ST, CC, capsular group and strain designation of 10 paired pharyngeal carriage isolates

The red font indicates the pair with different STs from the same subject.

ID	Visit	ST	CC	Capsule group	Strain designation
1A	2	ST-35	cc35	B	ND: P1.22-1,14: F4-1: ST-35 (cc35)
1B	6	ST-6798		**Y**	ND: P1.5-1,10-26: F5-5: ST-6798 ()
2A	2	ST-53	cc53	cnl	ND: P1.7,30: F1-2: ST-53 (cc53)
2B	3	ST-53	cc53	cnl	ND: P1.7,30: F1-2: ST-53 (cc53)
3A	4	ST-53	cc53	cnl	ND: P1.7,30-8: F1-6: ST-53 (cc53)
3B	5	ST_53	cc53	cnl	ND: P1.7,30-8: F1-6: ST-53 (cc53)
4A	3	ST-53	cc53	cnl	ND: P1.7,30-3: F1-2: ST-53 (cc53)
4B	5	ST-53	cc53	cnl	ND: P1.7,30-4: F1-2: ST-53 (cc53)
5A	4	ST-3651	cc22	W	ND: P1.5-1,2-2: F5-1: ST-3651 (cc22)
5B	6	ST-3651	cc22	W	ND: P1.5-1,2-2: F5-1: ST-3651 (cc22)
6A	3	ST-1157	cc1157	cnl	ND: P1.22,14: F5-36: ST-1157 (cc1157)
6B	5	ST-1157	cc1157	cnl	ND: P1.22,14: F5-36: ST-1157 (cc1157)
7A	3	ST-823	cc198	cnl	ND: P1.18,25-44: F1-18: ST-823 (cc198)
7B	4	ST-823	cc198	cnl	ND: P1.18,25-44: F1-18: ST-823 (cc198)
8A	2	ST-823	cc198	cnl	ND: P1.18,25: F5-5: ST-823 (cc198)
8B	5	ST-823	cc198	cnl	ND: P1.18,25: F5-5: ST-823 (cc198)
9A	2	ST-1167		B	ND: P1.22-1,14: F1-20: ST-1167 ()
9B	6	ST-1167		B	ND: P1.22-1,14: F1-20: ST-1167 ()
10A	2	ST-213	cc213	B	ND: P1.22,14: F3-7: ST-213 (cc213)
10B	3	ST-213	cc213	B	ND: P1.22,14: F3-7: ST-213 (cc213)

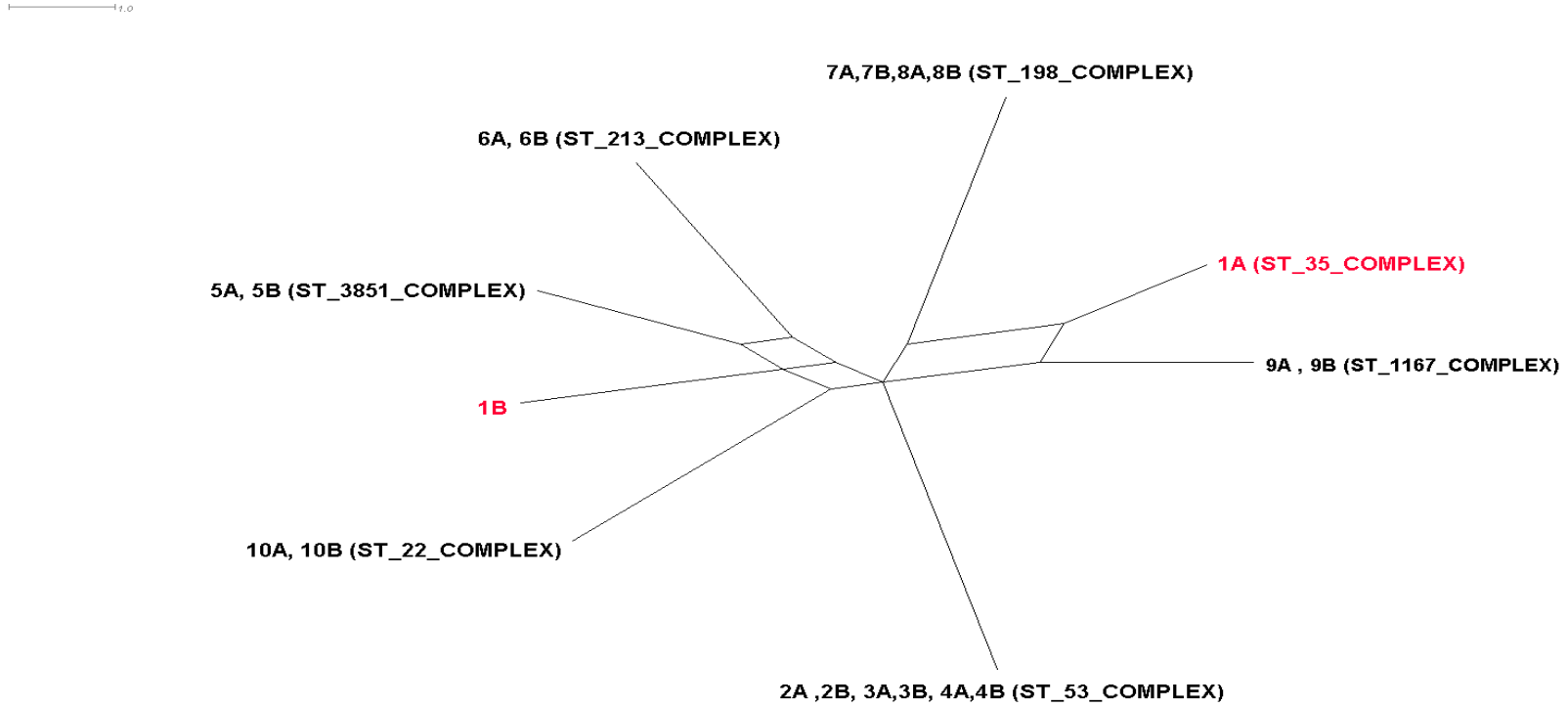


Figure 6.10 Phylogeny of paired meningococcal carriage isolates Split tree based on allelic differences in Neisseria meningitidis core genome MLST genes.

The number 1-10 indicates the ID from the paired samples, A and B indicates the different visits, The STs outlined in red indicate the discordant pair.

6.6 Discussion

Using WGS, the presence or absence of 47 Nm genes was assessed in BrisMenNHC carriage isolates for which gene expression analysis had been done using NanoString. The different clonal complexes, capsular genogroups, *fHbp* variants and peptides were identified in all carriage isolates.

Most genes were confirmed present in the genome of all isolates. Exceptions were (*frpC*) which was completely absent from all isolates, *nadA* which was absent in 70% of the isolates and the capsule synthesis genes (cssA, cssB and cssC) which were absent in isolates whose capsular group was either cnl or ND. Genes with highly variable sequences such as the pilin genes, which are known to have difficulty in genome assembly were scanned and partial sequences of those samples were found to be present in all isolates.

Therefore, most genes for which no expression was detected were probably switched off as opposed to absent in the genome. However, although the *nadA* gene was present in 30% (15/47) of the isolates, transcript counts for *nadA* were detected in 28/47 isolates. A potential explanation for the false positive gene counts for *nadA* is the known potential cross-reactivity of the *nadA* probe with other neisserial species including *N. gonorrhoeae*, *N. lactamica, N. elongata,* and *N. bacilliformis.* Bacteria of these species may have been present in pharyngeal samples (see Chapter 3.4.5) and this is a recognised limitation of this technique when testing samples from a non-sterile site that is likely to contain mixed bacterial populations. Indeed, the five highest *nadA* transcript counts were found in isolates that did not contain the gene, suggesting that these transcript counts were from cross-reactive organisms.

The *frpC* gene was absent from the genomes of all Nm isolates yet 8 samples had an expression result for this gene although mostly at levels of <50 gene counts/1000 bacteria. Similarly, expression of three capsule synthesis genes was seen in some cnl isolates, particularly *cssA*. All the three genes were absent in these isolates in addition to those with undetermined capsular group. As the probes did not show cross reactivity for these genes, this suggests that there is a low specificity of detection at low transcript counts or that other Nm were present that did not grow or get selected for sequencing. To improve specificity, one option would be to subtract the maximum count of the negative controls instead of the geometric mean. This should eliminate most false positive counts but would also mean that some true positives are missed.

Of the six IGR sequences related to the *fhbP* gene identified, 50% belonged to IGR allele number 1. IGR_1 has also been shown to be the predominate IGR in carriage isolates in previous studies (72). The variation of the IGR alleles was assessed by aligning the IGR sequences. The levels of *fhbP* expression in IGR_1 samples were consistent with expected levels from a previous study of expression among invasive strains of Nm(72). It was not possible to compare predicted against actual levels of *fhbP* expression among these carriage isolates by statistical analysis due to the small numbers of samples.

The WGS data were used to determine *fhbP* variants in the genome of the isolates and cross validate with the RT-qPCR results. Three of ten isolates had a discordant variant result, possibly because expression of *fhbP* in the carriage samples was below the detection limit by RT-qPCR, but this does show inaccuracy in some PCR results.

Forty-two isolates belong to five capsular genogroups (cnl, B, W, Y &E). No genogroup was identified for five isolates (ND). The majority of the BrisMenNHC carriage isolates

had no capsule synthesis genes (cnl), consistent with findings from previous carriage studies in Europe and Africa (145, 165, 172). The carriage isolates showed 22 different sequence types belonging to 13 clonal complexes. The predominant clone was cc1157.

Of the ten paired longitudinal isolates only one isolate had a different variant after a five-month interval. All the others had the same clonal complex, capsular group, *fhbP* variant and strain designation after intervals of 1-5 months. Of two sets of paired isolates with indistinguishable strains taken from two students at the same school, both students were male, age 17 years, and of white ethnic group from the same school year. These results suggest that carriage of Nm with the same strain may persist at least five months (the maximum time we tested). Similar results have been found in a study done in Italy on students aged 14-22 years (165) and in another study in Ethiopia (172). Modelling estimates have suggested that carriage can persist for over 12 months (173).

WGS was used successfully to characterise the isolates and assess the gene expression results obtained by NanoString. Some limitations of the technique were shown, particularly potential cross reactivity between different species in samples containing multiple organisms and possible false positive results at low levels of gene expression. Refinement of the cut off points may be needed to get the right balance between sensitivity and specificity at low levels of gene expression.

Chapter 7. General discussion and conclusion

Previous research in the field of Nm transcriptomics has relied on indirect evidence using different models to mimic the *in vivo* carriage transcriptional changes of Nm. This is the first study of the transcription profile of Nm carried out directly on *in vivo* carriage samples collected from healthy carriers. This thesis has presented the process of a sequence of optimisation of RNA extraction through detection of Nm gene transcripts from Nm cultures to detection of gene expression from *in vivo* carriage samples and validation of the methods. Strengths and limitations of the technique, and future development of this work will be discussed.

7.1 Summary of study findings

Optimisation of RNA extraction

As the RNeasy technique gave both a higher yield and higher quality of RNA than the alternative acid:phenol:chloroform method, the RNeasy minikit was used to extract RNA from Nm culture and pharyngeal swab samples. The use of bacterial lysate for gene expression analysis to avoid the process of RNA extraction was explored, however using undiluted RNA doubled the yield of RNA and had a higher purity. RNAlater was used for storage as it performed better than the alternative PrimeStore method.

Detection of gene expression using NanoString technology

The use of custom designed probe sets targeting 47 Nm genes plus three housekeeping genes established that Nm genes can be detected and counted from Nm ATCC samples cultured under different nutritional and temperature conditions.

The range of RNA detection from low and high bacterial density samples using different dilutions of RNA demonstrated the sensitivity of the NanoString technique. The high specificity of the probes was also confirmed using Nm negative controls. One of the important qualities of the NanoString platform should be its reproducibility. Running technical replicate samples and measuring gene expressions at different time points showed a high reproducibility with an R^2 of 0.99. The ability of the system to detect fold change in gene expression between control and test samples was also confirmed.

Transcript detection of gene expression of Nm from *in vivo* carriage samples, the first time that this has been attempted, was successful. All previous studies to try to understand gene expression of Nm in the pharynx had been done using cell lines (93, 95, 120, 174). Forty-seven Nm genes were detected in the CodeSet from pharyngeal carriage samples collected during three different studies. Genes expressed in relatively high quantity from the 47 selected genes were identified.

The NanoString nCounter system not only helped to assess the different degrees of gene expression, but also to assess the association of expression with pharyngeal carriage density. The density of bacteria carried in the nasopharynx is linked to risk of invasive disease for some organisms such as the pneumococcus (175, 176). It is not known if this is true for Nm. Interestingly, bacterial carriage density had a negative association with expression of many of the genes assayed. Although unexplained, it should be emphasized that all samples assayed were at a relatively high level of density, so that the association between expression and density could only be assessed among samples with >100 gene copies/ml. It is possible that at higher densities the bacteria form microcolonies and become partially dormant, or that there

is a mix of live and dead bacteria since the density measurement is of bacterial DNA as opposed to live colonies and that the lower expression reflects this.

Validation

Gene expression results for one gene *(fhbP*) from NanoString were compared with gene detection in the same Nm isolates by RT-qPCR. There was a good level of consistency between the two gene expression platforms (72% concordance), but there were some discordant results. This variability could be due to technical errors in either of the two platforms. Manipulation of RNA samples before performing RT-qPCR (double digestion to remove gDNA to avoid false positive results) means however that RNA can be lost or degraded in the digestion process, and the sensitivity of RT-qPCR in detecting very small quantities of RNA may be low. For the NanoString platform, specificity may be lower in samples with low gene counts. WGS showed that some genes that were absent from the genome were measured as having low levels of gene expression.

There was an association between the expression of *fhbP* with the different clonal complexes and IGR type, a finding consistent with other recent research (72). The clonal complexes and their capsular genogroups were identified, as well as the duration of carriage with the same strain over several months from the longitudinal BrisMenNHC samples.

7.2 Strengths

This is the first time that Nm genes have been profiled from *in vivo* Nm pharyngeal carriage samples. A total of 47 Nm genes was measured, including the expression pattern of 4CMenB protein vaccine genes. This could give an insight into which genes

are predominantly expressed and could generate protective immunity against carriage. For example, one the vaccine genes, *nadA*, was not found in most samples indicating that antibodies to this protein would be unlikely to protect against carriage. Proteins from genes expressed in many individuals in abundance could be potential vaccine targets.

The NanoString platform has a great advantage over other techniques in assessing the expression of Nm gene transcript abundance by directly counting each transcript as opposed to other techniques, such as microarray, that rely on relative counts or fluorescent based values. Another advantage of NanoString is the ease of sample preparation with minimal hands-on time that reduces the risk of technical errors compared to other gene expression platforms. The need to avoid purifying total RNA to get rid of gDNA is advantageous since RNA can be lost or degraded in the process of gDNA digestion. Potential errors in other techniques include the need to use multiple enzymes such as reverse transcriptase could introduce technical variation. The advantage of assessing many genes in a single reaction in an almost fully automated platform is that this reduces the chance of committing technical errors. The use of internal spiked positive and negative controls is a useful check on quality of the transcript count unlike other gene expression platforms.

7.3 Limitations

Potential limitations with the NanoString nCounter system were found during the course of these studies. It was not possible to design a fully species-specific probe since Nm shares many genes between species. Of the 47 genes selected for this study, 17 showed cross reactivity with other neisseria species. This is a major disadvantage when measuring gene expression from Nm in mixed samples where

other neisseria species may be present, for example, the detection of nadA in some samples where it was not present in the Nm genome.

Although it is possible that more than one colony was selected for WGS, another limitation with the Nanostring technique seen in this study was the observation that specificity was reduced at low levels of gene expression, showing for example eight false positive results for *frpC* when this gene was absent on WGS. Threshold cut off levels may need refinement.

It was only possible to analyse samples with bacterial density greater than 100 gene copies/ml. Although most Nm carriage samples had bacterial density <100 gene copies per ml, these samples may be of less importance for transcriptomic study as high-density samples are probably more relevant to the risk of transmission.

7.4 Implications and future work

Despite the limitations of the NanoString platform, its strengths allow an important insight into the expression of current or potential vaccine antigens. Highly expressed genes in many individuals could be used to propose new vaccine candidate proteins. Consistently expressed specific genes in our panel would help to pinpoint the active genes in carriage and transmission. Correlation of those consistently expressed genes with levels of immunity produced could be assessed in future work. The NanoString designed probes can be used to measure gene expression in disease isolates to compare with expression pattern in carriage isolates. The technique has the potential for study of gene expression in carriage and disease isolates of other pathogenic organisms carried in the human pharynx such as *Streptococcus pneumoniae, Staphylococcus aureus,* and *Haemophilus influenzae.*

7.5 Conclusion

The methodologies established in this thesis could be very important for future transcriptomics related studies on Nm and other bacteria carried in the human pharynx. The potential of the NanoString nCounter technology to assess different genes from complex samples, such as pharyngeal samples even where bacterial density is relatively low, will give an insight into the biology of Nm transcriptomics. This is especially relevant to the assessment of actual and potential vaccine antigens. One of the current protein vaccine genes (*nadA)* even if it had been highly immunogenic, is not expressed in most Nm carriage strains, so that the effect of this protein in a vaccine to generate herd protection may be limited. Other proteins that are expressed more consistently and at higher levels may have greater potential as vaccine candidates.

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Appendix A Gene detection and expression profiling of *Neisseria meningitidis* using NanoString nCounter platform

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Journal of Microbiological Methods

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Note

Gene detection and expression profiling of Neisseria meningitidis using NanoString nCounter platform

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ARTICLEINFO ABSTRACT

Detection of bacterial gene transcripts in low density mucosal samples is challenging. We evaluated the NanoString nCounter system for transcript detection in Neisseria meningitidis (Nm) cultures. The method was sensitive, reproducible (R^2=0.99) and demonstrated changes in gene expression. Studying Nm transcripts from pharyngeal samples may be feasible using this approach.

Keywords:
Transcriptomics
NanoString
Gene expression
Neisseria meningitidis
mRNA

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Full text available from: https://doi.org/10.1016/j.mimet.2018.02.003

Appendix B The 47 selected genes with their functions

Vaccine antigens	
Gene/protein	Comment/ Function
Components of Bexsero 4CMenB vaccine	
In general, these vaccine antigens were identified through reverse vaccinology as conserved proteins that generate bactericidal antibodies	
fhbP/fHbp (also in Trumenba)	Factor H binding protein. Surface exposed lipoprotein which binds to human factor H, an inhibitor of the alternative complement pathway.
nadA / NadA G	Neisseria adhesin A. Pathogenicity factor with a role in host cell adhesion and invasion.
nhbA/NHBA	Neisserial heparin-binding antigen. Lipoprotein which binds heparin in-vivo through an arginine rich region and protects against complement.
porA /PorA	Variable but important in immunity. Component of OMV NZ
GNA2091 (Combined with fHbp)	Induced protective immunity in some assays.
GNA1030 (Combined with nhba)	Induced protective immunity in some assays.
Capsular polysaccharide vaccines	
cssA, cssB, cssC	Capsule biosynthesis for genogroups B, C, W, Y.
Potential vaccine antigens	
Proposed as vaccine candidates. In general, there is less certainty about ability of these antigens to generate bactericidal antibodies in humans and/or they are less well conserved.	
nspA/NspA	Neisserial surface protein A. Binds to domain 6 and 7 of human fH and acts cooperatively with fHbp. Potential vaccine antigen. However, phase 1 trial showed no bactericidal antibodies.
tbpA&tbpB/TbpA&TbpB	Transferrin binding protein. Potential vaccine antigen. Uncertainty around bactericidal immunity.
fetA(frpB) /FetA	Iron-regulated meningococcal outer membrane protein. Potential vaccine antigen

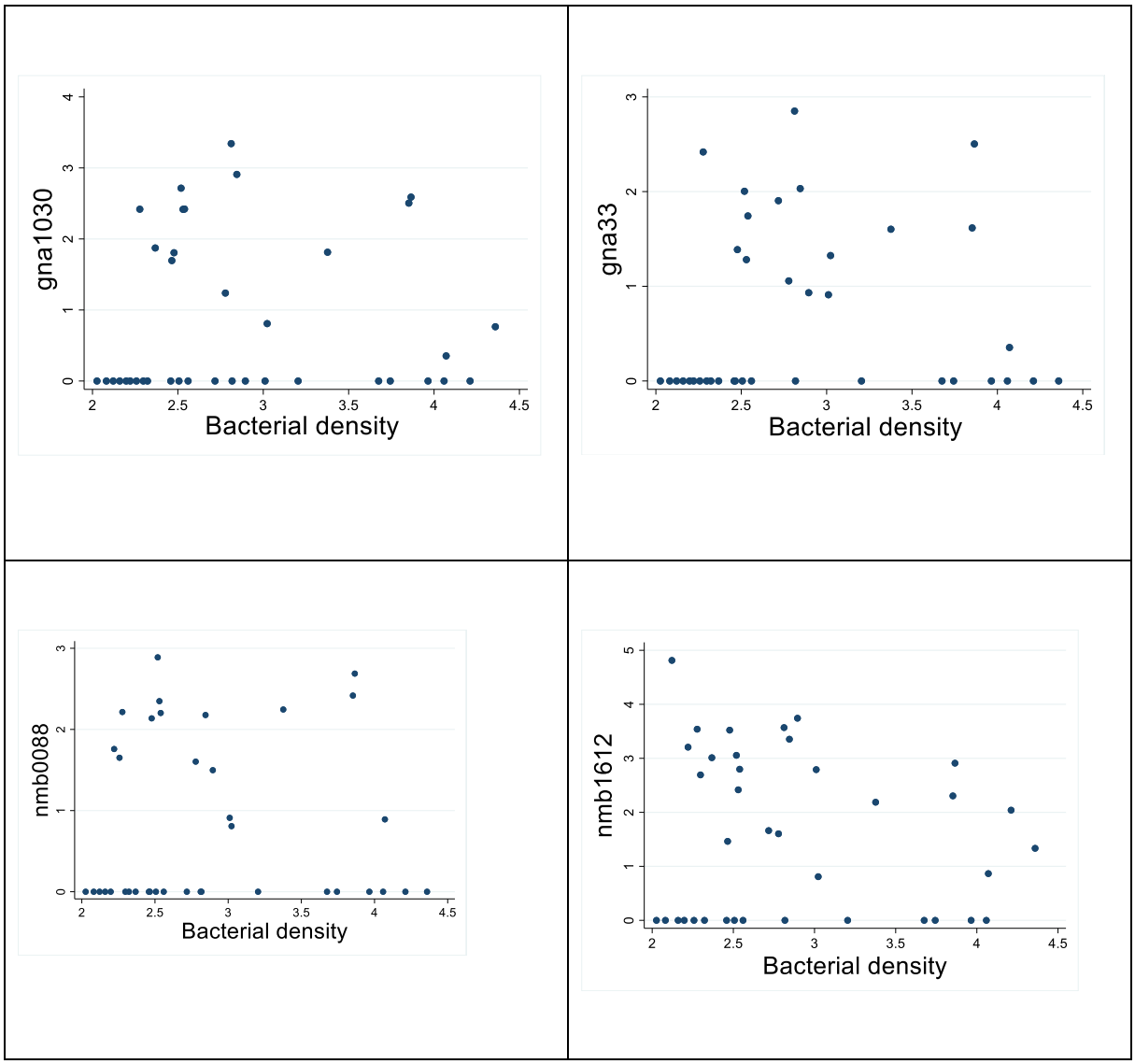
opc/opCA	Major role in adhesion. Binds to serum vitronectin to inhibit complement mediated killing. Potential vaccine antigen. Can generate bactericidal antibodies
NMB 1567/ MIP	Macrophage infectivity potentiator. Proteomics identified as potential vaccine antigen. Important for meningococcal survival in blood. Can elicit bactericidal antibodies in mice but not in all studies.
NMB 1946	Proteomics identified as potential vaccine antigen. Can elicit bactericidal antibodies in mice.
NMB 1468	Uncharacterised protein. Proteomics identified as potential vaccine antigen. Can elicit bactericidal antibodies in mice
NMB 1612	Metabolic amino acid ABC transporter. Proteomics identified as potential vaccine antigen but may not generate bactericidal antibodies.
NMB 0088/ FadL like protein	Proteomics identified as potential vaccine antigen. Can elicit bactericidal antibodies in mice.
pilP/PilP	Proteomics identified as novel potential vaccine antigen. Tfp biosynthesis and motility via interaction with OMP secretion pilQ.
pilE/PilE	Proteomics identified as potential vaccine antigen. Main constituent of Type IV pili, but variability has prevented use in vaccines.
NMB0294 /DsbA-2	Proteomics identified as novel potential vaccine antigen. Important in disulphide bond formation.
GNA33	Lipoprotein, lytic transglycolase. Genome derived neisserial antigen identified through reverse vaccinology. Highly conserved. Elicits bactericidal antibodies. Potential vaccine candidate.
GNA992	Adhesin. Genome derived neisserial antigen identified through reverse vaccinology. Conserved, elicits bactericidal antibodies. Potential vaccine antigen (26)
tspA/TspA	T cell stimulating carrier protein A (51). It may enhance the effectiveness of vaccines by acting as protective immunogen (25)
acp/ ACP	Adhesin complex protein. Upregulated under iron-depleted conditions Vaccine potential
Role in colonisation	

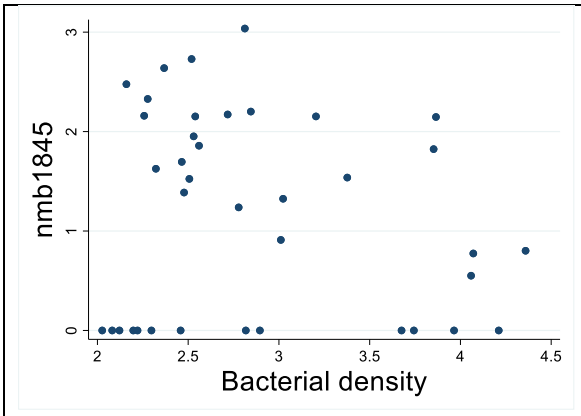
Many potential genes. Selection on basis of importance, virulence, variation in expression during colonisation. Probably not considered as vaccine candidates.	
msf/Msf	Meningococcal surface fibril. Previously called nhhA or hsf which binds preferentially to activated vitronectin (aVn).
pilC/PilC1	Required for pilus-mediated adhesion.
xseB	Inducible repair system. Upregulates when interacts with host cells. Critical for adaptation to host
NMA0797	Two component system required for colonisation. Promotes adaptation of Nm to grow on host cells.
narP/NarP	Two-component system. Metabolism of nitrogen oxides: encoding the NarP regulator of the two-component system NarP/NarQ, involved in denitrification process. Identified using in-vitro colonisation model.
nnrS/NnrS	Encodes heme- and copper-containing membrane protein.
NMB0552	Identified using in-vitro colonization model.
estD/ esterase D	Putative esterase D. Identified using in-vitro colonisation model.
fadD1	Long chain fatty acid-CoA ligase. Identified using in-vitro colonisation model.
NMB1845	Putative membrane associated thioredoxin. Identified using in-vitro colonisation model.
cysW	Permease. Induction following adhesion.
cysT	Permease. Induction following adhesion.
cysA	Sulphate ABC transporter. Induction following adhesion.
NMB0573	Controls the response to nutrient availability, important role in colonisation, indicator of general amino acid abundance.
pilN/PilN	Adhesin. Involved in assembly of Type IV pili. Virulence gene upregulated on colonisation.
pilO/PilO	Adhesin. Involved in assembly of Type IV pili.
pilV/PilV	Adhesin. Involved in assembly of Type IV pili.
nhhA/NhhA	Neisseria hia/hsf homologue A (also known as hsf). Auto-transporter like NadA. Facilitates attachment to epithelial cells.

app/App	Adhesion and penetration protein. Auto-transporter like NadA.
nadR/NadR	Transcriptional regulator. Binds nada promoter.
hsp90/Hsp90	Heat shock protein. Interacts with NadA.
iga/ IgA	Immunoglobulin IgA protease. Virulence gene upregulated on colonisation.
fbpB/ FbpB	Involved in iron uptake. Virulence gene upregulated on colonisation.
tonB/ TonB	Involved in iron uptake. Virulence gene upregulated on colonisation.
lbpB/ LpbP	Involved in iron uptake. Virulence gene upregulated on colonisation.
expB/ ExpB	Iron uptake. Virulence gene upregulated on colonisation.
opa/Opa	Major role in adhesion and invasion. Integral outer membrane protein synthesized as a precursor, contains signal for inner membrane transport. Virulence gene upregulated on colonisation.
frpC/ FrpC	Potential toxin. Virulence gene upregulated on colonisation.
hpuA /HpuA	Iron acquisition. Lipoprotein, haemoglobin receptor. Shows phase variation in persistent meningococcal carriers.
hmbR /HmbR	Involved in the catabolism of Heme, hem. Shows phase variation in persistent meningococcal carriers.
mspA /MspA	Autotransporter. Shows phase variation in persistent meningococcal carriers.
nalP /NalP	Autotransporter. Shows phase variation in persistent meningococcal carriers.
fur	Iron regulator in Neisseria meningitidis. Responds in low iron environment.

Appendix C Scatter plot of *Neisseria meningitidis* transcript count (log) vs. bacterial density(log) showing expressed and unexpressed genes

The x-axis is bacterial transcript count (log) and the y-axis is the transcript count in gene copies/ml (log).

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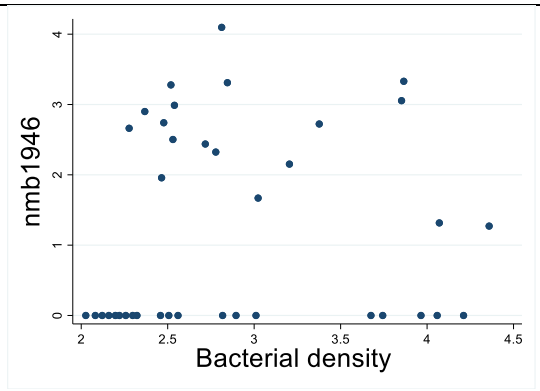
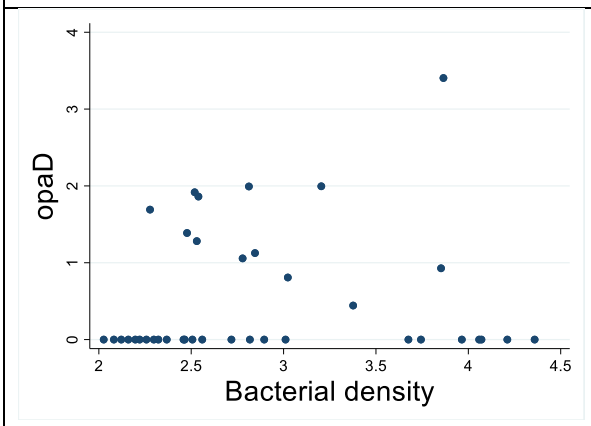
Bacterial density


nmb1946
Bacterial density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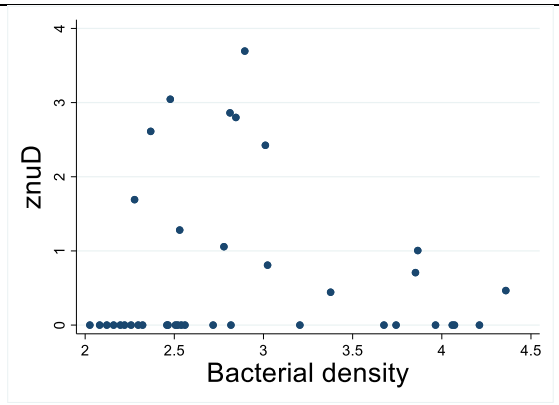
Bacterial density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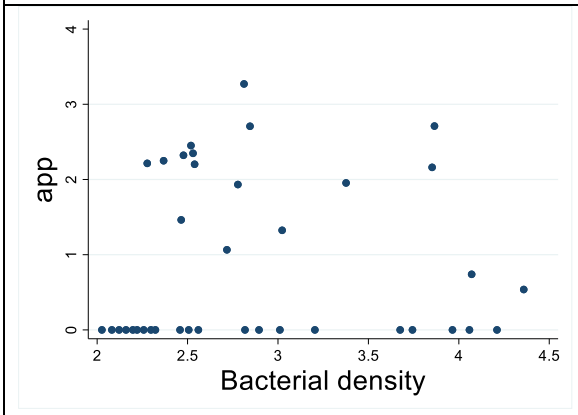
Bacterial density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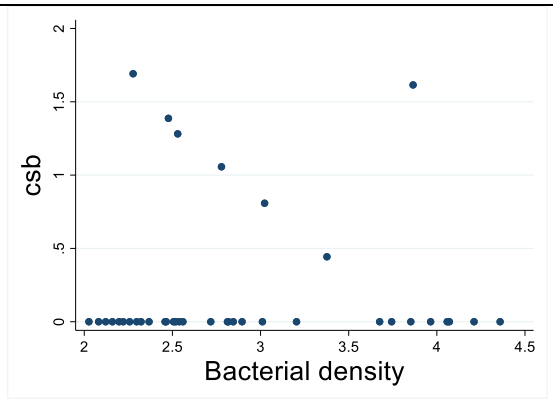
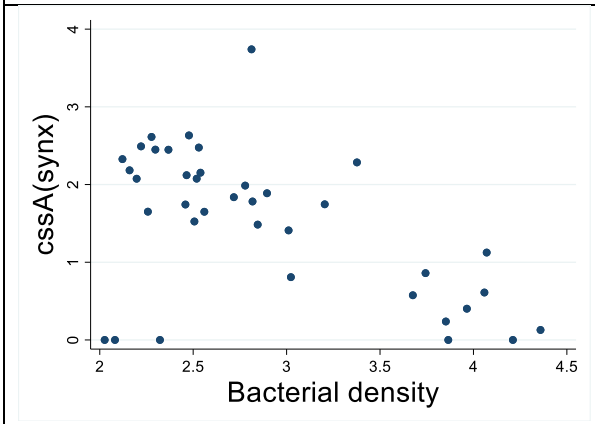

csb
Bacterial density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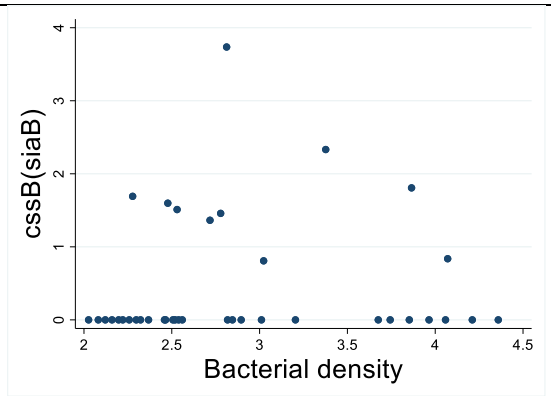
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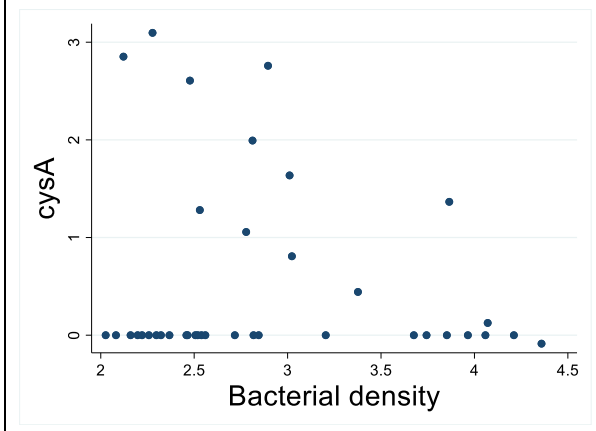
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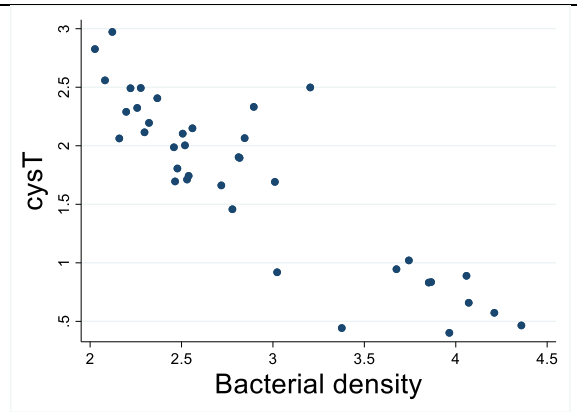
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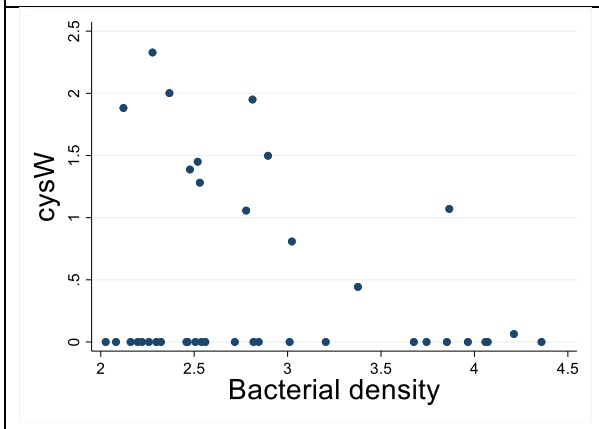
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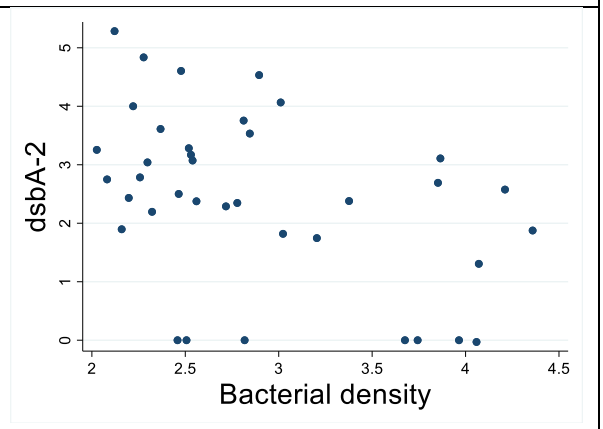
Bacterial density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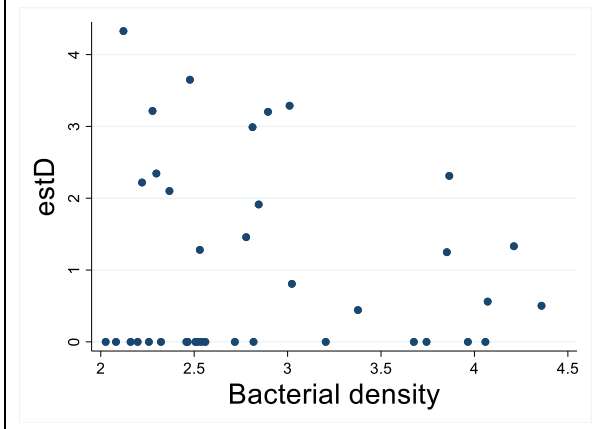
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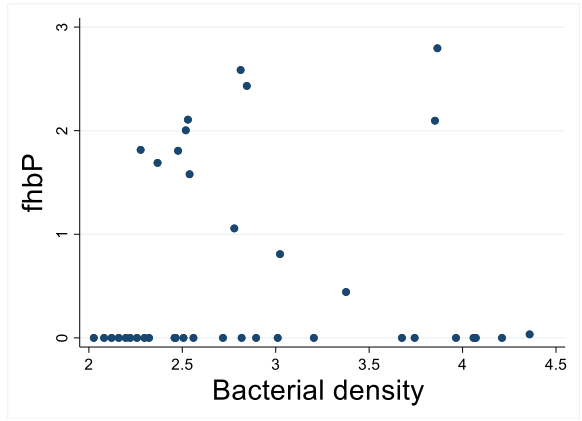
Bacterial density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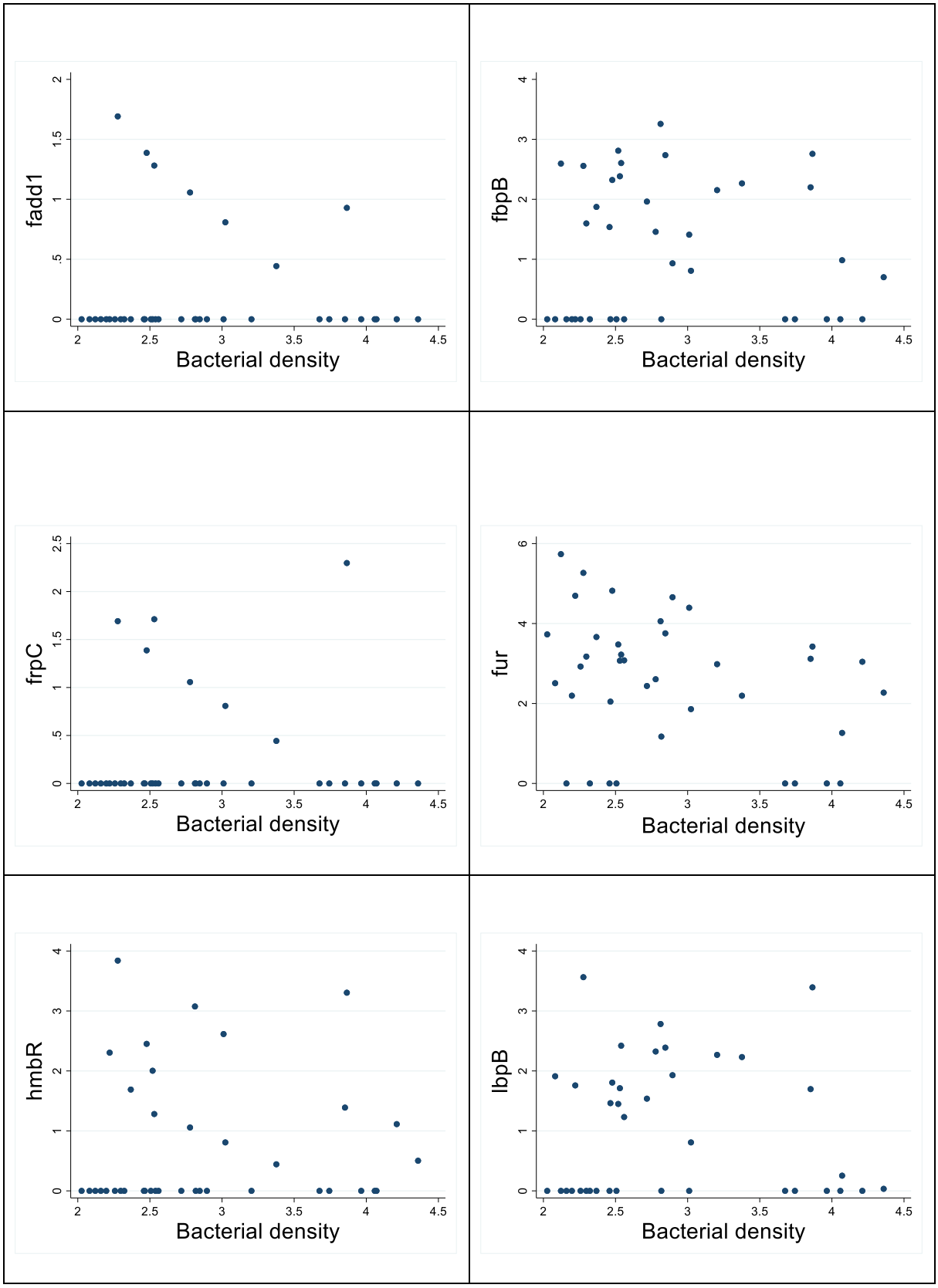
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frpC
Bacterial density
fur
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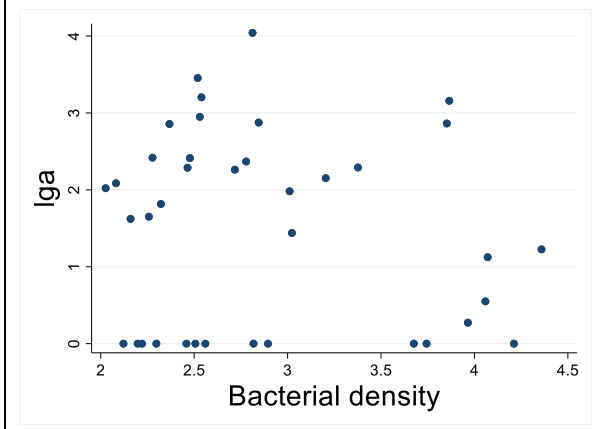
hmbR
Bacterial density
lbpB
Bacterial density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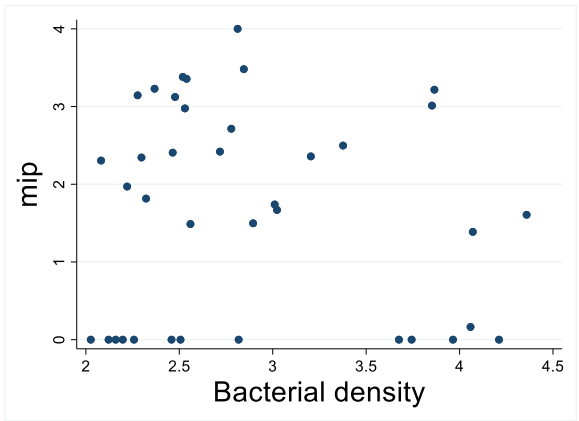
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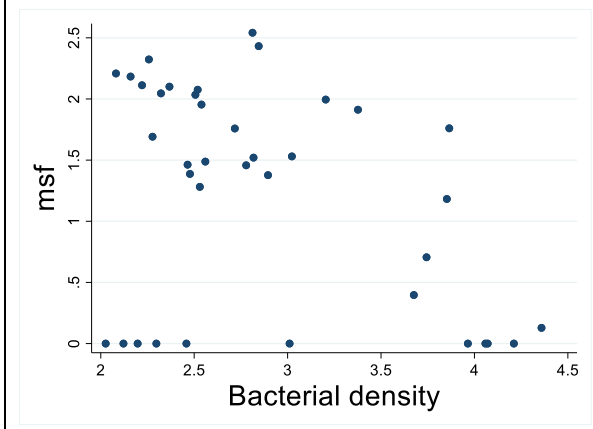
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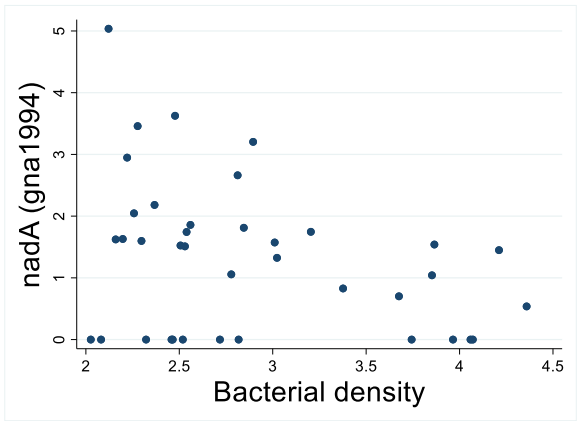
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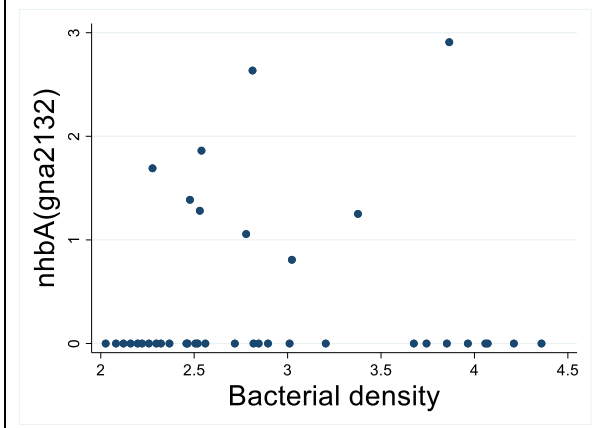
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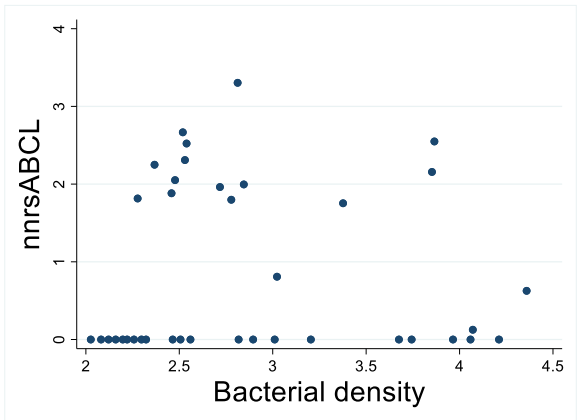
Bacterial density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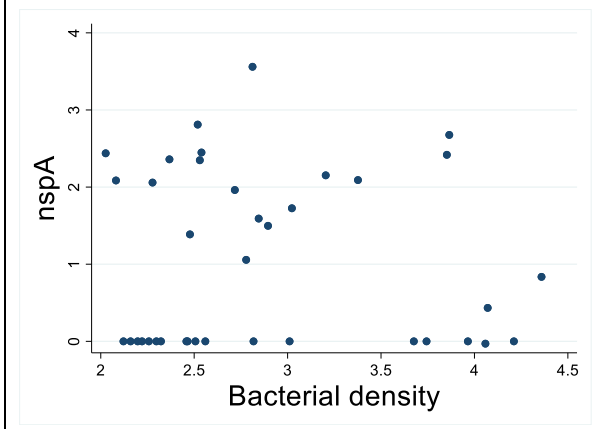
Bacterial density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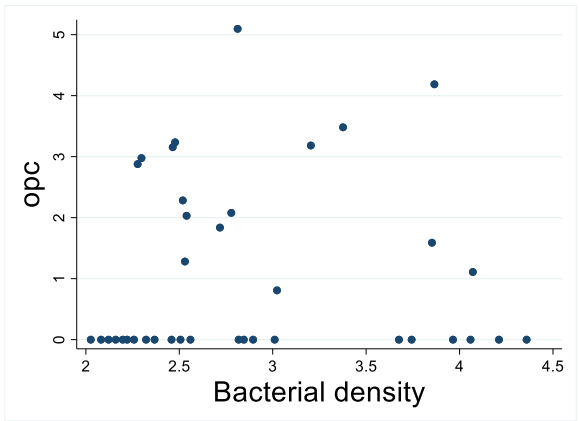
opc
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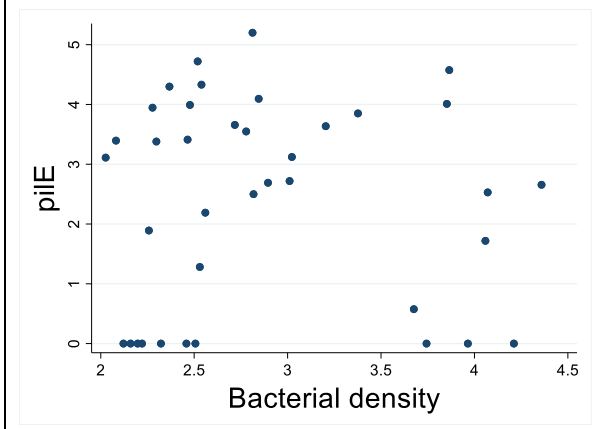
Bacterial density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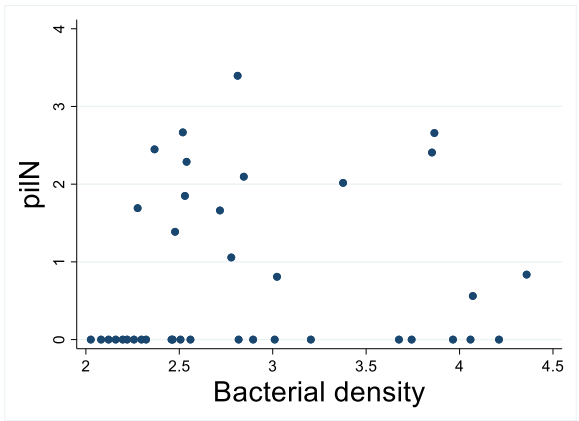
Bacterial density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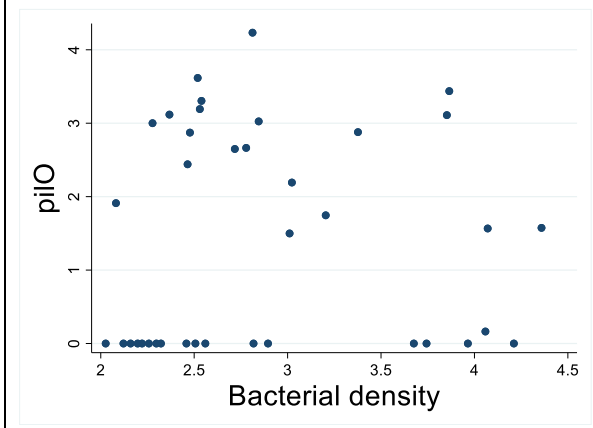
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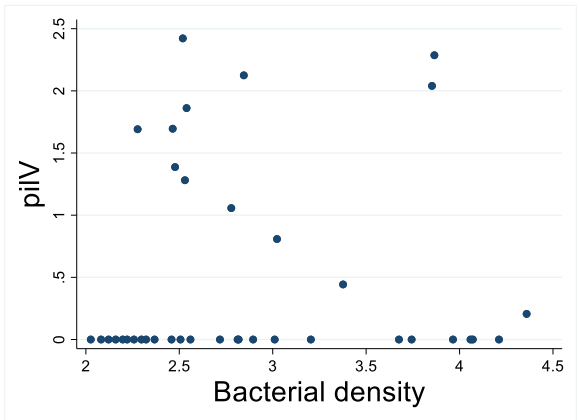
Bacterial density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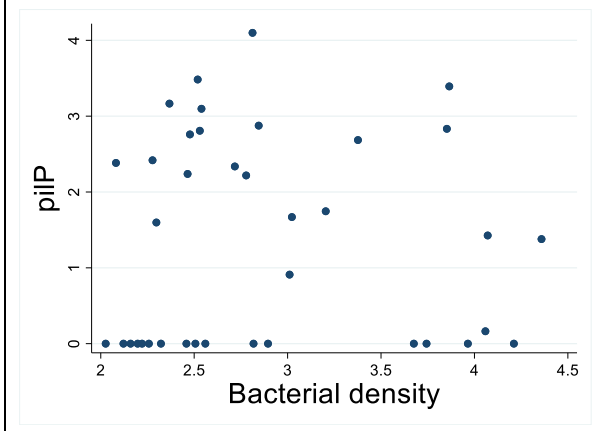
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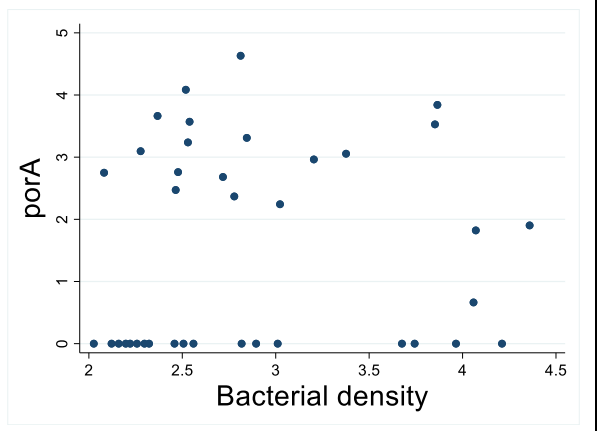
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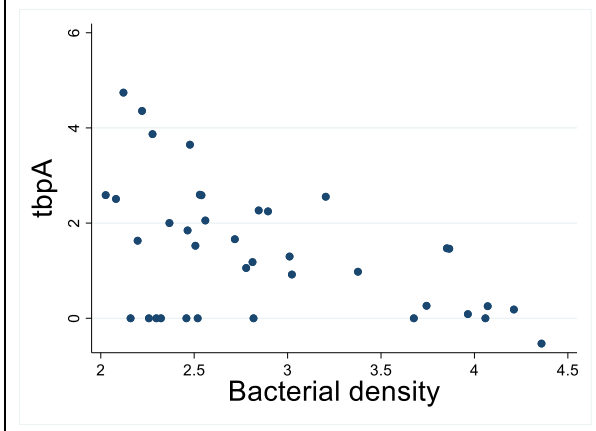
Bacterial density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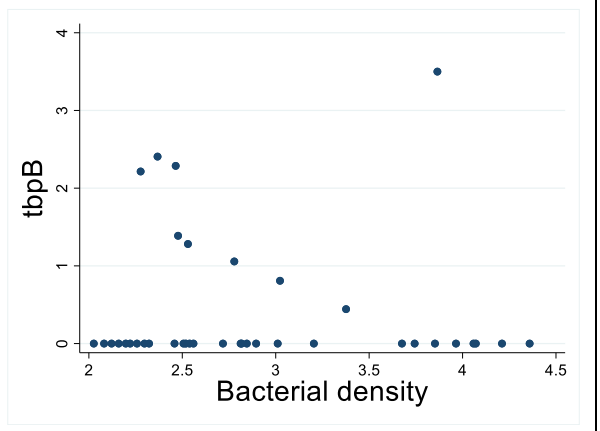
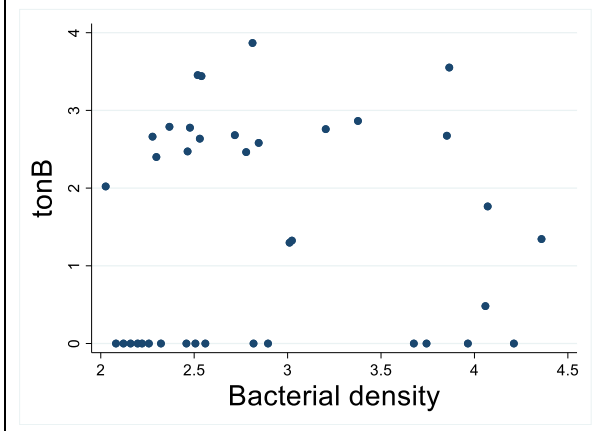
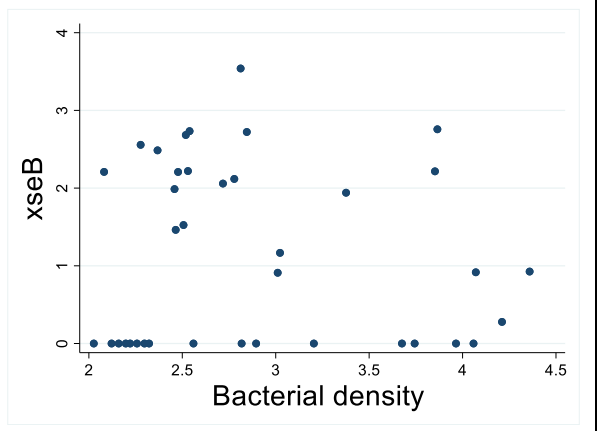
Bacterial density


tbpB
Bacterial density


tonB
Bacterial density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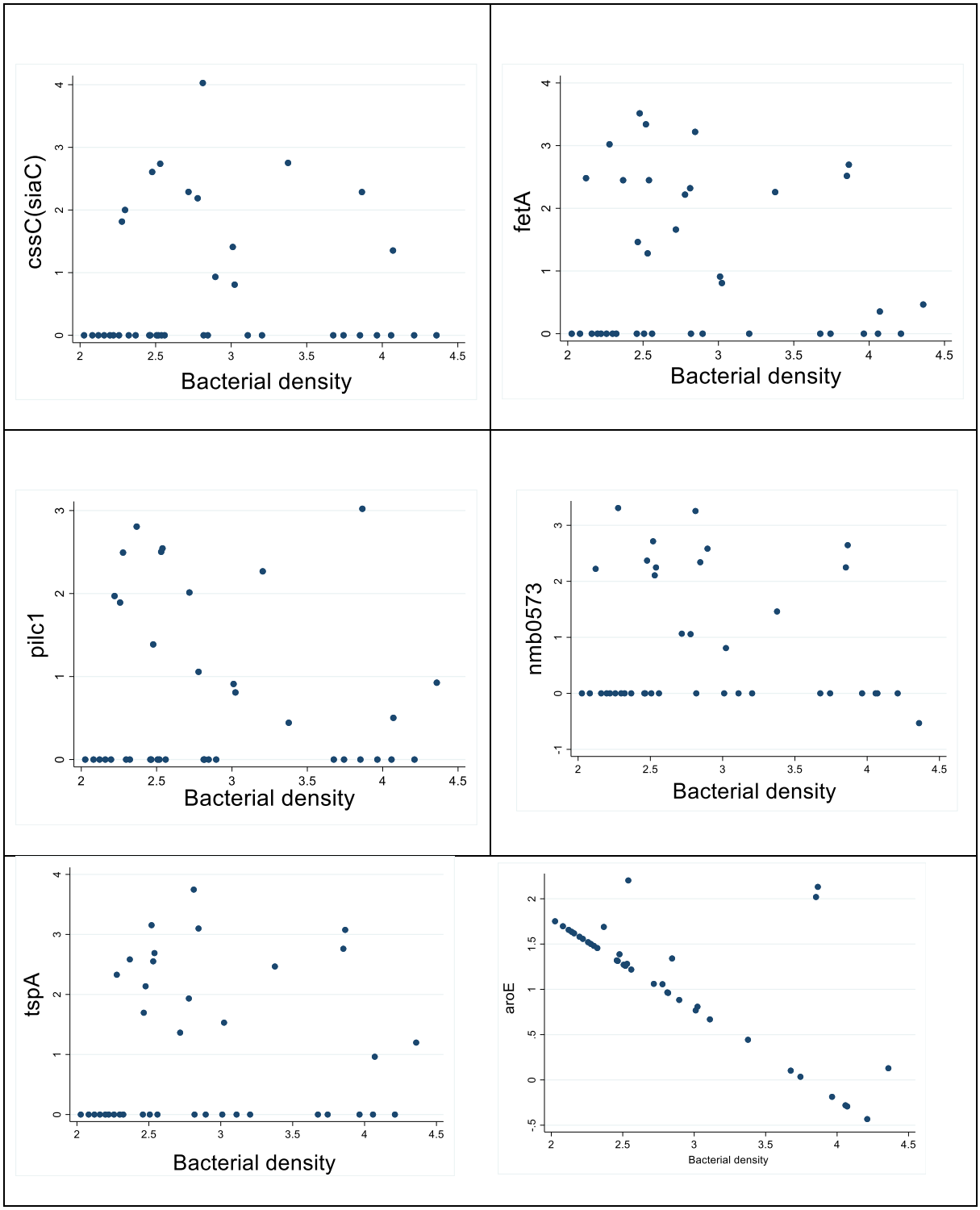
Bacterial density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Bacterial density
fetA
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pilc1
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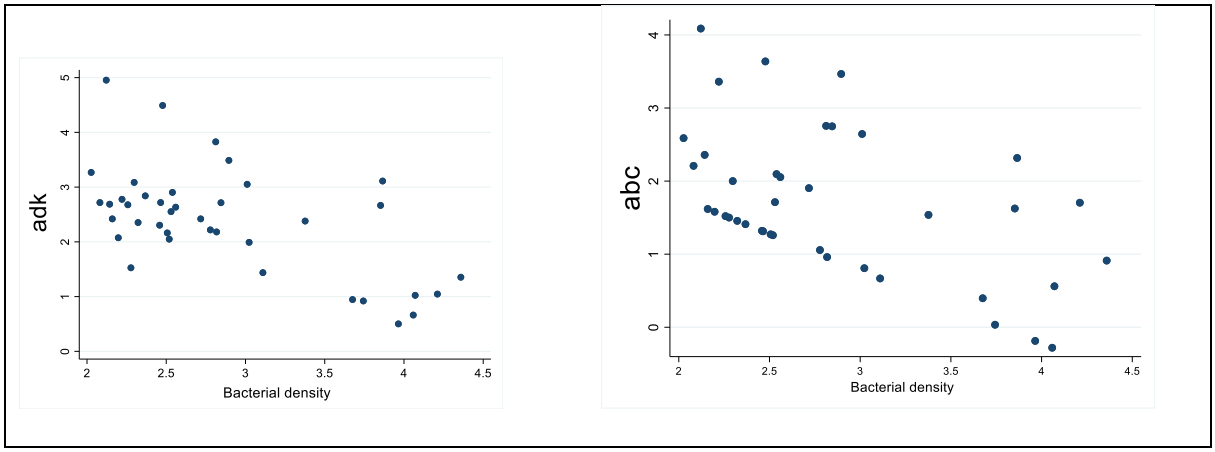
aroE
Bacterial density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Bacterial density
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2
2.5
3
3.5
4
4.5
Bacterial density

Appendix D Protocol BrisMenNHC study

Recruitment and follow up.

Any participants who indicated, during consent to the cross-sectional Wellcome Trust funded study, that they would be happy to be approached about a further study will be asked to provide their contact details. Ethics-approved information about the study will be distributed to students at participating schools/colleges in collaboration with those institutions. Student and school staff engagement and participation in organisation of recruitment will be welcomed whenever offered. Consenting and swabbing sessions will be organised at the schools.

Consent will be taken in writing from students by members of the study team at the time of enrolment (first follow-on swabbing visit). The majority of eligible students are expected to be between 16 and 18 years old; however, a few may be 15 or 19 years old. Participants aged 15 can give consent for themselves as long as they fully understand the proposed procedure (Gillick competent). In such cases there is a degree of clinical judgement, which would be exercised by the study team members involved in taking consent.

Students will be asked to complete a brief questionnaire and have a throat swab taken at each visit. Communication with participants concerning follow up visits will be guided by the participating schools but, based on experience of previous studies, will most likely be conducted by SMS-text messaging and email.

Collection of pharyngeal swabs

After consent has been obtained, the oropharynx of each participant will be swabbed by a trained member of the research team. A sterile double-headed oropharyngeal

swab will be used to briefly touch the back of the throat, sweeping from one tonsil to the other across the back of the pharynx, behind the uvula. Some people find this uncomfortable, and a small proportion will 'gag' when the swab is taken; this is a harmless reflex and is only momentary.

One "head" of the double-headed swab will be introduced into a 2ml vial containing 1.5 ml skimmed milk-tryptone-glycerol-glucose (STGG) broth, the shaft broken, and the swab tip left inside the vial. The other "head" of the swab will be placed into a 2ml vial containing 1.5 mL RNAlater solution. All samples will be kept at 2-8°C and returned to the laboratory within 2-6 hours. There they will be vortexed, and the STGG vials frozen at minus 80°C until analysis. These samples will be used for detection of meningococci by PCR. (Subject to further funding, they could also be used for detection of meningococci by culture.) The RNAlater samples will be incubated overnight at 4°C to allow thorough penetration of the swab tissue by the solution and will then be transferred to -80°C for long-term storage. All samples will be collected, used, stored and disposed of in accordance with the Human Tissue Act 2004.

Meningococcal PCR.

A *sodC*-based multiplex real-time PCR assay will be used for the detection of Neisseria meningitidis. Primer and probe sequences will be based on published literature. (177) PCR reaction volume will be 20µl using a 384-well optical plate. Each PCR reaction mixture will contain 5µl of sample DNA extract, 5µl of primer/probe mix (100µM concentration) and 10µl of TaqMan® Fast Universal PCR Master Mix (Applied Biosystems®, California, USA). The assay will be carried out using the Applied Biosystems® ViiA7™ Real-Time PCR system under the following conditions: 1 cycle of enzyme activation at 95°C for 20 seconds, then 50 cycles of denaturation and

annealing at 95°C for 3 seconds followed by elongation at 60°C for 60 seconds. T4 bacteriophage will be used as an internal control to ensure adequate DNA extraction with 10µl added prior to the DNA extraction process. Negative controls will be included in every 24-sample batch. N. meningitidis ATCC 53417 reference strain will be used as positive controls and dilutions will be included in each RT-PCR run.

Analysis will be performed using ViiA7™ Software version 1.1 (Applied Biosystems®, California, USA). Fluorescence measurements will be taken in real time and for each sample the cycle threshold (Ct) will be taken as fluorescence detectable above the baseline level. Ct values of ≤36 will be taken as positive.

Conversion of cycle threshold (CT) numbers to colony forming units (CFUs). For the bacterial quantitative PCR assay, N. meningitidis ATCC 53417 reference strain is used. The organism is cultured in liquid medium (BHI agar supplemented with 10% defibrinated horse blood and 2% isovitalex enrichment) and retrieved in the exponential growth phase (assessed using spectrophotometry). The solution is serially ten-fold diluted in Skimmed milk-Tryptone-Glucose-Glycerol (STGG) broth and plated out for counting of colony forming units (CFUs). The same dilution series is used for real time bacterial PCR for conversion of cycle threshold (CT) values to CFU/ml. For negative control, the PCR assays is run on blank STGG broth and a panel of non-targeted bacterial species. DNA extractions from the target species are used as positive controls. The standard curve for N. meningitidis is based on an average of pooled data of two dilution series, each run three times, and this curve is used to create a conversion equation from CT values to CFUs/ml.

Appendix E Protocol SPIT study

Recruitment, immunisation and follow up

Information about the study will be distributed to students at participating schools/colleges in collaboration with those institutions. Student and school staff engagement and participation in organisation of recruitment will be critical to success and preliminary contact has already been established. Consenting, vaccination and sampling sessions will be organised at the schools. Students will indicate their interest/willingness to participate by attendance at these clinics and will have the opportunity to ask questions and discuss the study with a member of the research team before consent is taken.

Consent will be taken in writing from students by suitably trained members of the study team at the time of enrolment. Participants will be asked for permission to inform their GP of their participation in the study, and to check GP and/or Child Health Database records for any other meningococcal vaccines they have received. Consent will also be sought for storage and future research on left-over samples after the study analyses have been completed; this will be optional, and students will be able to take part whether or not they agree to this aspect. The majority of eligible students are expected to be aged between 16 and 18 years, although some may be older.

Following consent, participants will be asked to complete a brief questionnaire on risk factors for meningococcal carriage and to provide a saliva sample. Students will undergo a health check by a nurse at the start of the study to ensure they are fit to receive the vaccine. All female participants will be assessed for the possibility of being pregnant.

Vaccines will be administered by suitably trained and experienced nurses. Participants will be monitored immediately after receiving vaccine and asked to report any side effects they are concerned about. Equipment for the treatment of anaphylactic reactions will be available in the unlikely event that such a reaction occurred. Throat swabs will be taken before each vaccine dose and at the final study visit. At the second vaccination visit, participants will be asked to complete a brief questionnaire to evaluate acceptability of the study methods and to elicit comments and suggestions for future studies. They will be asked to provide weekly saliva samples (during term-time) from enrolment, including at the first and second vaccinations, and again at the final study visit (3-5 months later).

Communication with participants concerning follow up visits will be guided by the participating schools but, based on experience in previous studies, will most probably be conducted by primarily SMS-text messaging and email.

Sample collection

Pharyngeal swabs

The oropharynx of each participant will be swabbed by a trained member of the research team. A sterile oropharyngeal swab will be used to briefly touch the back of the throat, sweeping from one tonsil to the other across the back of the pharynx, behind the uvula. Some people find this uncomfortable, and a small proportion will ‘gag’ when the swab is taken; this is a harmless reflex and is only momentary.

One “head” of the double-headed swab will be introduced into a 2ml vial containing 1.5 ml skimmed milk-tryptone-glycerol-glucose (STGG) broth, the shaft broken, and the swab tip left inside the vial. All samples will be kept at 2-8°C and returned to the laboratory within 2-6 hours. There they will be vortexed, and the STGG vials frozen at

minus 80°C until analysis. These samples will be used for detection of meningococci by PCR of DNA extracts (for density estimation) and/or DNA extracts of lawn cultures (for maximum sensitivity and optimal genogrouping). The other swab “head” will be stored in RNA preservation medium for future transcriptomics studies (to evaluate microbial and host gene expression profiles associated with carriage acquisition, density and clearance). The RNAlater samples will be incubated overnight at 4°C and will then be transferred to minus 80° C for long-term storage.

All samples will be collected, used, stored and disposed of in accordance with the Human Tissue Act 2004 and no future studies will be undertaken on specific samples in the absence of appropriate and explicit consent.

Saliva samples

Participants will be provided with an empty 2ml vial, asked to pool saliva in their mouth, and then project it into the vial until this is at least half full. Saliva will either be stored neat or added to an equal volume of STGG broth prior to storage thus allowing culture amplication prior to PCR.